Influence of growth rate on the immature skeleton

BY
DIANNE HAMILTON MURRAY, BSc (HONS).

Thesis Presented For The Degree Of Doctor Of Philosophy
In the
Faculty Of Medicine & Veterinary Medicine Of The University of Edinburgh
2005

DECLARATION

I declare that this thesis was composed by myself and has not been presented in any previous application for a degree. Help given by others has been acknowledged and all sources of information have been specifically referenced.

Dianne Murray

ACKNOWLEDGEMENTS

I would like to thank Professor Hamish Simpson and Dr Colin Farquharson, my supervisors for this project. In particular, Dr Colin Farquharson for obtaining the initial funding from BBSRC and his support and encouragement throughout. A big thank you must go to Avigen, Midlothian, for their supply of day old chicks, without which this project would of been impossible. I would also like to acknowledge British Poultry Science for travel funding to the ECTS conference in Rome 2003 and the Biochemical Society for travel funding to the ASBMR conference in Seattle 2004.

Thanks to Dave Waddington and Heather McCormack for their patience while I attempted to grasp the fine art of statistics. Thanks also must go to Nigel Loveridge who was always at the end the phone with his support and advice on various aspects of the project. Advice and help were received from many of the staff in our department at Roslin Institute. Special thanks to Bob Fleming for advice and help with image analysis packages and polarised microscopy. Thanks to Elaine Seawright for my introduction to cell culture and Heather McCormack and Lynn Mcteir for their time and help during injection administrations. Thanks to Graham Robertson for the instruction in confocal microscopy, and a huge thanks to the staff of the poultry department who looked after my chickens.

Finally, I would like to say a big thank you to my partner, family and friends who were always there with support and encouragement.

List of Abbreviations

ALP	alkaline phosphatase
ARF	activation, resorption and formation
BMU	basic multicellular unit
BrdU	5-bromo-2-deoxyuridine
BSP	bone sialoprotein
CMFDA	celltracker green
CSMI	cross sectional moment of inertia
dH_2O	distilled water
EthD-1	ethidium homodiner-1
FBS	fetal bovine serum
FGF	fibroblast growth factor
GLAST	glutamate/aspartate transporter
IGF-1	insulin-like growth factor -1
LCM	laser capture microscopy
MCSF-1	macrophage colony stimulating factor-1
NMDA	N-methyl-D-aspartate (glutamate receptor)
NO	nitric oxide
NOS	nitric oxide synthase
eNOS	endothelial nitric oxide synthase
iNOS	inducible nitric oxide synthase
nNOS	neuronal nitric oxide synthase
ODF	osteoclast differentiation factor

OHP	hydroxyproline
OPN	osteopontin
OPG	osteoprotegrin
OPGL	osteoprotegrin ligand
PBS	phosphate buffered saline
PTH	parathyroid hormone
RANK	receptor activator of nuclear factor-kappa β
RANKL	receptor activator of nuclear factor-kappa β ligand
RT	room temperature
TRAP	tartrate resistant acid phosphatase
TNF	tumour necrosis factor

PUBLICATIONS AND PRESENTED ABSTRACT IN SUPPORT OF THESIS

REFEREED ARTICLES

1 Williams BG, Waddington D, **Murray DH**, Farquharson C (2004). Bone strength during growth: Influence of growth rate on cortical porosity and mineralization. Calcified Tissue International 74 (3):236-245

CONFERENCE ABTRACTS

1 **Murray DH**, Williams BG, Waddington D, Farquharson C (2002). Bone strength during growth: Influence of growth rate on cortical bone porosity and mineralisation. IX Congress of the International Society of Bone Morphometry Edinburgh, Edinburgh J. Bone Min. Res. 17:946.

2 **Murray DH**, Loveridge N, Williams BG, Waddington D, Farquharson C (2002). Influence of growth rate on cortical bone porosity, and strength in the immature skeleton. Bone and Tooth Society, Cardiff. Bone and Tooth Society, Cardiff. J. Bone Min. Res. 17:1324

3 **Murray DH**, Williams B, Loveridge N, Waddington D, Farquharson C (2002). Osteocyte formation and reduced bone strength during rapid growth. 24th Amer. Soc. Bone Min. Res. San Antonio, USA . J. Bone Min. Res. 17:S242.

4 **Murray, DH.,** Loveridge, N. & Farquharson C (2003). Cortical porosity during fast growth in the immature skeleton:the role of periosteal osteoblast proliferation. Bone and Tooth Society Annual Meeting, Sheffield, July 9-11 2003. Journal of Bone and Mineral Research Vol. 18 7, pp 1367.

5 **Murray, DH.**, Loveridge, N., Waddington, D., Farquharson C (2003). Cortical porosity during fast growth in the immature skeleton: The role of periosteal osteoblast proliferation and differentiation rates. 30th European Symposium on Calicified Tissues, 8th-12th May, 2003, Rome, Italy. Calcified Tissue International Vol. 72, pp 437, abstract P-421.

6 **Murray DH**, Williams BG, Waddington D, Farquharson C (2003). Bone formation during rapid growth in the immature chicken:effects on mechanical properties, mineralisation and cortical porosity. Comparative Endocrinology of Calcium Metabolism Symposium June 2-3, Osaka, Japan.

7 **Murray DH** and Farquharson C (2004). Osteoblast characterisation and gene expression during fast growth in the immature skeleton. Bone and Tooth Society Annual Meeting, Oxford, June 29-30.

8 **Murray DH** and Farquharson C (2004). Osteoblast Gene Expression Profiles During Growth of the Immature Skeleton. 26th Amer. Soc. Bone Min. Res. Seattle, USA, Oct 1-5.

Summary

Bone architecture adapts to withstand the loads placed upon it. In response to increased loads during growth, bones circumferentially expand to increase their diameter through the incorporation of periosteal blood vessels and the formation and infilling of primary osteons. However, the influence of growth rate on bone architecture in the immature skeleton is not fully understood.

To investigate how bone architecture is modulated by growth rate, morphometric, biochemical and genetic comparisons were made between tibiae from broiler chickens with either fast or slow growth potentials.

Both strains of chickens were kept under identical conditions, and fed ad-libitum standard broiler feed. Tibiae were removed and tested by three-point bending to determine stiffness and breaking strength and cross sections of the tibia were examined histomorphologically. Bone stiffness and breaking strength were higher in the rapidly growing birds, but after adjustment for body weight the bones were inherently weaker. Cortical porosity periosteally, but not endosteally, was increased. Sections reacted for ALP and TRAP activity, and others stained for cement (reversal) lines indicated the absence of primary osteon remodelling in the periosteal region. This suggests that the increased periosteal porosity is due to slower infilling of the primary osteons in the rapidly growing birds. To directly quantify the rate of osteon infilling, tibiae were removed from 21-day-old chicks, which had been double labelled with calcein (80 and 8 h before death). The mineral apposition rate was higher in the slow growing chickens, and confirmed the previous histomorphometry results. Osteocyte density within the circumferential lamellae of the cortical bone

was higher in the rapidly growing birds but unchanged within the newly laid down bone of the primary osteons.

Immunohistochemical staining of cortical bone sections from chickens injected with bromodeoxyuridine located proliferating pre-osteoblast cells to the osteogenic layer of the periosteum. A lower labelling index in the rapidly growing birds was seen across four circumferential areas of the periosteum (anterior = fast growing area, posterior = slow growing area, medial and lateral = intermediate growing areas), even though the osteogenic layer of the periosteum was thicker in the fast strain. Blood vessel numbers within the periosteum was similar between strains but differed between regions habitually loaded in tension (anterior) or in compression (posterior).

Osteoblasts were grown and expanded in culture from explants of tibia cortical bone (periosteum removed) of 21-day-old birds of both strains (n=4/strain). Osteoblast proliferation was determined by tritiated-thymidine uptake and differentiation by alkaline phosphatase (ALP) activity. At pre-confluency, cell proliferation was higher in the slow growing birds, but this pattern was reversed at confluency and post confluency which was a likely consequence of impairment of proliferation by contact inhibition in the slow growing strain. ALP activity was only detected at post-confluency and was higher in the fast growing strain. Osteoblastic gene expression was determined by RT-PCR and quantified by densitometry. A higher level of osteopontin, and bone sialoprotein expression (BSP) was observed in the slow growing birds. Whereas Runx2 and the serotonin receptor, considered to have a role in mechanoregulation, were more highly expressed in the fast chickens.

In conclusion, fast growth resulted in the expected circumferential expansion to increase bone bending strength. Fast growth was accompanied by increased porosity

resulting from the rapid formation of primary osteons and the incapacity of osteoblasts to completely infill the resultant canal. However, periosteal interstitial bone of the fast growing birds had a higher osteocyte density suggesting that the lack of infilling was not due to a decrease in osteoblast number. No evidence was obtained to suggest that osteonal remodelling or periosteal blood vessel number were a determinant for primary osteon size. However, the lower labelling index at the periosteum and increased osteocyte density within the circumferential lamellae of the fast strain suggests an increase in transit time through the osteoblast lineage at the periosteal surface. In vitro, osteoblast proliferation was faster in the slow growing birds whereas differentiation was slower. This is in accord with the previous hypothesis that the fast growing birds are characterised by an increase in transit time through the osteoblast lineage, which may be driven by the high levels of Runx2 expression. Osteopontin and BSP are associated with mechanical loading but the significance of the lower expression levels in the fast growing birds requires further study. However, the up regulation of serotonin expression may reflect the greater loads experienced in the fast growing birds in vivo.

CONTENTS

Chapter 1: Bone, a literature review and thesis aims. **PAGE**

Chapter 2: Materials and Methods

Chapter 3: Determination of the cause of increased cortical porosity in the fast growing broiler. A histomorphometric study.

Chapter 4: Determination of the cause of decreased osteonal infilling in the fast growing broiler. A histomorphometric and immunocytochemical study

Chapter 1: Bone, a literature review and thesis aims.

1.1: General introduction

Bone function

Bone is a highly specialised connective tissue providing support and mechanical integrity, and supply of inorganic ions associated with mineral homeostasis (Bab and Einhorn 1994). To adequately fulfil structure and function relationships, bone is constantly being broken down and rebuilt in a process known as remodelling. The cellular link between bone forming cells; osteoblasts, and bone resorbing cells; osteoclasts, is known as coupling. Bone formation and resorption are linked, but when the balance is upset between the two, this leads to disease. Under normal states of bone homeostasis bone will be laid down where needed, such as at the areas of high mechanical loading/strain, and removed from areas where there is a low mechanical need. Moreover, in the immature skeleton, bone modelling is highly significant, where upon the long bones must adapt to the growing body. This is done by customising the geometry of the bones as the animal grows, adapting to the continually changing mechanical load placed upon it. Therefore, bone as a specialised connective tissue, is well designed with the ability to process mechanical and physiological signals and transfer these into cellular and chemical reactions.

1.2: Bone composition

Bone is composed of two phases, organic and inorganic. By weight approximately seventy percent is mineral/inorganic, twenty-two percent extracellular matrix/organic, and the final eight percent water. Of the mineral phase ninety-five percent of this is composed of crystalline hydroxyapatite, (Rosenberry et al 1931) which with its endogenous impurities make up the final five percent. Ninety-eight percent of the extracellular matrix is composed of Type I collagen, with the final two

percent being made up of noncollagenous proteins and cells, (Einhorn 1996). The extracellular matrix of bone plays the role of determining the structure, mechanical and biomechanical properties of the tissue. A high proportion of mature cortical bone by weight is collagen, with approximately eleven percent being non-collagenous organic material (Herring 1972), such as proteoglycans, glycoproteins, plasma proteins, peptides and lipids.

1.2.1: Collagen

Collagen occurs in all multicellular animals and is the most abundant protein of vertebrates making up approximately thirty percent of the body protein. It is organised into insoluble fibres of great tensile strength which suits collagen in its role as the major stress bearing component of connective tissues such as bone, teeth, cartilage, tendon, ligament and fibrous matrices of skin and blood vessels (Voet and Voet 1995). It has also been recognised as having several biological roles such as cell attachment (Kleinman et al 1981), platelet aggregation (Morton et al 1987), morphogenesis and development (Adamson 1982) and chemotaxis (Postlethwaite et al 1978) amongst others. A collagen molecule is 300nm long, with a triple chain helix, containing 1011-1042 amino acid residues per polypeptide chain (α chain), with the triple helical structure being responsible for its characteristic tensile strength. Each chain has a repeating amino acid triplet sequence of Gly-X-Y, where the X and Y residues are frequently proline and hydroxyproline, with hydroxyproline restricted to the Y position because of the specificity of prolyl hydroxylase. The repetitive triplet structure is essential for the helical formation, with glycine being the only amino acid small enough to be accommodated in the interior of the helix (Robins 1988). The presence of proline and hydroxyproline in the α chains increases

the stability of the triple helix. The variety of other amino acids which occupy the X and Y positions decrease the helix stability, but are essential for the assembly into fibrils (Vaughan 1981). There are at least fifteen different variations of the molecule resulting from genetic polymorphism in the collagen molecule, with the major forms appearing in bone and cartilage being type I and type II, respectively. The collagen I molecule in bone is composed of two slightly different types of α chains with different amino acid sequences, these being α_1(I) (2 chains), and α_2(I) (1 chain) (Robins 1988). Collagen type I and II are synthesised by osteoblasts and chondrocytes, respectively as part of longer pro-collagen molecules containing additional pro-peptides. The pro-collagen molecules are firstly secreted into the matrix, where further processing cleaves the pro-collagen molecule leaving the collagen molecule, which spontaneously assemble into fibrils (Fig 1.1). New collagen fibres are stabilised by cross-links formed by the action of lysyl oxidase on lysine and hydroxylysine residues, eventually maturing into trivalent structures: pyridinoline, incorporating a hydroxylysine residue, and deoxypyridinoline incorporating a lysine residue (Farquharson et al 1989, Calvo et al 1996). The ratio of the pyridinoline to deoxypyridinoline in bone is 4:1 (Eyre et al 1988).

Within the collagen fibril there are gaps called ''hole zones'' between the end of one fibril and the start of a new fibril. It is within these gaps that non-collagenous proteins or minerals are found and mineralisation of the extracellular matrix is thought to start from these points, (Lodish et al 1996). The mineral phase is mainly composed of calcium and phosphorous, forming a crystalline hydroxyapatite, present as a plate like structure.

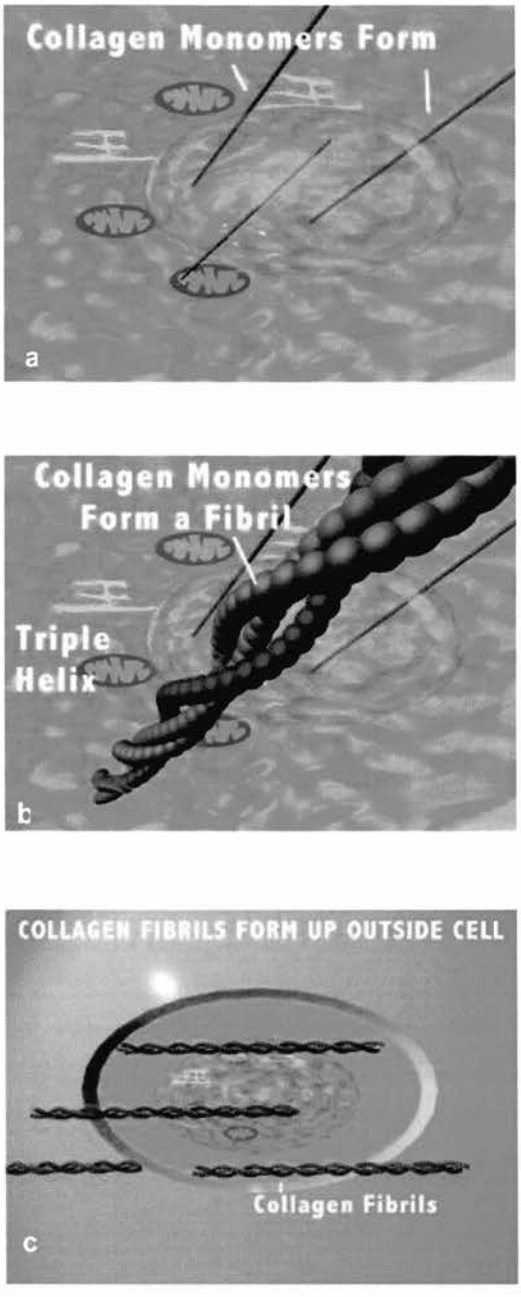


Figure 1.1 Collagen monomers are formed (a), which combine into a collagen fibril triple helix (b), and these collagen fibrils form up outside the cell (c).

1.2.2: Non-collagenous Proteins

1.2.2.1: Osteopontin (BSP-1)

Osteopontin (OPN) is a major non-collagenous protein found in bone, it is an acidic glycoprotein and is also found in breast milk, serum, endometrial glands of secretory phase uterus and several tissues of the developing ear (Butler 1989, Young et al 1990). OPN is an acidic protein of about 300 amino acids of 44 kDa in size (Butler 1989, Miyazaki et al 1990). The overall amino acid homology between several species is high, including a stretch of seven to nine residues of Asp-Glu, which may represent a calcium or hydroxyapatite binding site. OPN is known to bind covalently to collagen type I and to fibronectin via transglutamination, and it is this transglutamination which increases the OPN binding to collagen type I (Beninati et al 1994). OPN has the ability to mediate cell attachment suggesting it could determine tissue organisation and cell migration, and the high negative charge on the protein may help create a permeability barrier, acting as a buffer to calcium levels (Denhardt and Prince 1993). Study of OPN at the cellular level during sub-periosteal bone formation, indicates that it is produced by osteoblasts making it a late marker of osteoblastic differentiation, and an early marker of mineralisation (Strauss et al 1990, Robey et al 1992). It has been suggested that osteocytes also express OPN (Noble and Reeve 2000), when they are exposed to mechanical stress (Terai et al 1999). Osteoclasts also express OPN when absorbing bone in osteoarthritis (Dodds et al 1995) with OPN being observed underneath the clear zone involved in the attachment of the osteoclast to the bone matrix (Reinholt et al 1990). OPN expression is regulated by mechanical stress, whereas OPN itself is involved in bone resorption and possibly bone formation, and therefore suggests OPN plays one or more roles in the response to mechanical loading (Noda and Denhardt 2002). OPN has also been reported to be an inhibitor of hydroxyapatite formation (Hunter et al

1994), by blocking crystal elongation (Boskey et al 1990) thus inhibiting mineralisation.

1.2.2.2: Bone sialoprotein (BSP-II)

Bone sialoprotein (BSP) is a heavily glycosylated extracellular matrix protein, making up ten to fifteen percent of the non- collagenous proteins in the mineral compartment of developing bone. Phosphorylated non-collagenous proteins are found in all vertebrate mineralised tissue and have been hypothesized to play a role in initiating or controlling the distribution of mineral in the extracellular matrix (Glimer 1989). Numerous phosphorylated polypeptides have been found in chicken bones, ranging from 6-150 KDa (Lee and Glimer 1981, Uchiyama et al 1986, Ymazuki et al 1989), and data suggests that at least two genetically distinct phosphoproteins are present in chicken bone (Uchiyama et al 1986, Ymazuki et al 1989, Gotoh et al 1990). A glycosylated phosphoprotein with molecular weight mass of 44 KDa was found to be osteopontin (Fisher et al 1990, Gotoh et al 1990), and the second phosphorylated protein isolated with a molecular weight of 60 KDa was bone sialoprotein (Fisher et al 1987, Gotoh 1990). In man and cow, BSP protein contains about fifty percent carbohydrate (12% is sialic acid) and stretches of polyglutamic acid (polyaspartic acid in osteopontin), and has no disulphide bonds (Fisher et al 1983, 1987). Both rat and human (Oldberg et al 1988, Fisher et al 1990), cDNA show an Arg-Gly-Asp (RGD) tripeptide which is known to support cell attachment through the integrin receptor (Rouslshti and Pierschbacher 1987). Rat BSP binds to the vitronectin receptor (Oldberg et al 1988) and human BSP supports cell attachment through RGD mechanism in vitro (Somerman et al 1988). BSP has many of the properties of the cell attachment domain of fibronectin or vitronectin. It

appears to be relatively specific to the skeleton and found in osteoblasts, osteocytes, osteoclasts and hypertrophic chondrocytes, and supports cell attachment and cell-cell interactions (Fisher 1993). BSP is relatively unique to mineralising tissues, (Oldberg et al 1989, Fisher et al 1990) expressed only by cells forming a mineralised matrix, with expression highest during early matrix mineralisation (Ibaraki et al 1992). This, together with the observation that it acts as a hydroxyapatite nucleator (Hunter et al 1993) has lead to the opinion that BSP is considered to be involved in the mineralisation process.

1.2.2.3: Osteonectin

Osteonectin, a prominent constituent of bone; is a glycoprotein which binds Ca^{2+}, and is 32 kDa in size. It is also known as ''secreted protein, acidic and rich in cysteine'' (SPARC) (Mason et al 1986). Osteonectin has the ability to complex with collagen type I and hydroxyapatite to nucleate hydroxyapatite deposition within bone (Termine et al 1981), but has also been found expressed in non-osteogenic tissues (Sage et al 1984, Wasi et al 1984). Varying amounts of osteonectin are found in different mineralised tissues (Tung et al 1985), and in the same mineralised tissues of different species (Zung et al 1986), indicating that the protein associates with preformed mineral in amounts that reflect access to the mineral by the protein (Domenicucci et al 1988). The protein sequence determined from the mouse species has ten exons and four distinct domains (Engel et al 1987, McVey et al 1988). The first domain is formed with exon three and four encoding for an N-terminal domain which has two glutamate rich segments which can bind more than eight Ca^{2+} ions. The second domain is formed from exons five and six, which is rich in disulphide bonds and gives stability to the protein. The third domain is formed from exons

seven and eight and is predicted to have an α-helical conformation susceptible to proteolysis. The fourth domain is formed from exon nine and ten which contains a single high affinity EF-hand which binds Ca^{2+} with a characteristic helix-loop-helix structure (Engel et al 1987). It is considered that all Ca^{2+}-binding sites will be fully occupied at physiological concentrations of Ca^{2+} and therefore osteonectin is unlikely to be involved in Ca^{2+} regulation (Engel et al 1987). Osteonectin can compose up to fifteen percent of the non-collagenous proteins in bone depending on the developmental age and the animal species. Expression in adult tissue is limited to cells associated with mineralised tissue such as osteoblasts, hypertrophic chondrocytes, and odontoblasts, and knock-out mice deficient in osteonectin develop osteoporosis (Delany et al 2000).

1.2.2.4: Osteocalcin

Osteocalcin was the first bone matrix protein to be isolated and makes up approximately 15 % of the non-collagenous protein in bone. However this level varies depending on the animal species (Conn and Termine 1981). The protein has a molecular weight of 5.3 KDa, and contains one disulfide bond and three-five residues of γ-carboxy glutamic acid, with the number dependent on animal species (Hauschka et al 1975). Osteocalcin is synthesised by both osteoblasts and osteocytes and is considered to be a marker of osteoblastic function (Aronow et al 1990) as well as a coupler for osteoblast-osteoclast action. Osteocalcin has also been reported to be important for induction of the osteoclast phenotype (Liggett et al 1994). Osteocalcin is expressed late in osteoblast differentiation, making it difficult to identify transcription factors acting at early stages.

1.3: Bone organisation

1.3.1: Growing bones

The skeletal system incorporates cartilage, ligaments, tendons, and other structures which hold the bones together and stabilise them. The skeleton is composed of two types, the axial skeleton incorporating vertebrae, pelvis and other flat bones, and the appendicular skeleton incorporating all long bones. Growing long bones, (Fig 1.2) formed by a process called endochondral ossification are comprised of three anatomical regions, epiphysis, metaphysis, and diaphysis. The epiphysis is at both proximal and distal ends of the bone. It is separated from the rest of the bone by growth cartilage. Below and juxtaposed is the metaphyseal region which in turn is joined to the main body of the bone; the diaphyseal region. It is the metaphyseal region where development and remodelling takes place (Einhorn 1996).

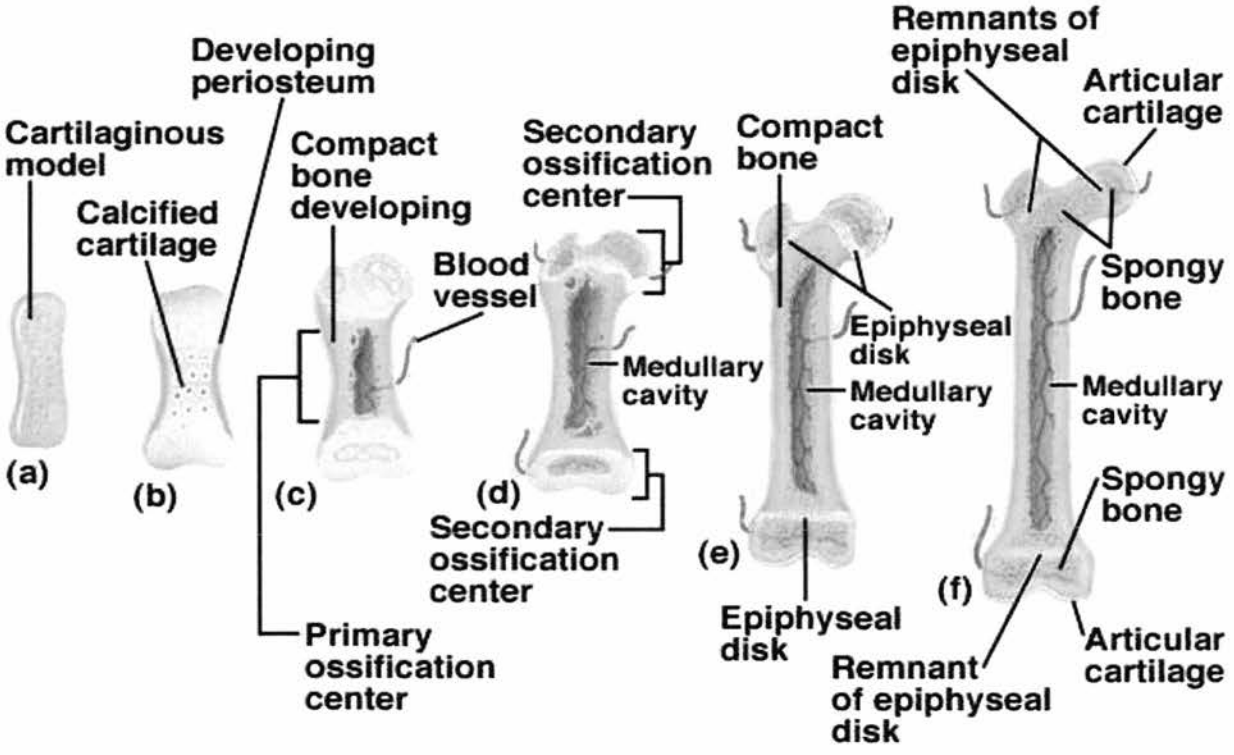


Figure 1.2 Endochondral bone development of long bones. Source fire.biol.wwu.edu/lapsansk/ 348/endoch_bone.jpg

1.3.2: Woven and Lamellae Bone

There are two types of bone, woven and lamellar. Woven bone is new, primitive or immature bone, found at growing areas of the skeleton, within the embryo and newborn, at fracture sites and within certain bone tumours (Einhorn 1996). Woven bone is course fibred with no uniform orientation of the collagen fibres. The osteocyte cell number per unit volume of bone is higher than that of lamellar bone, with the mineral content and cells being randomly arranged. This is replaced later by the more permanent lamellar bone. Woven bone is also associated with rapid bone formation and a special type of this, medullary bone, is present in the marrow cavity of egg-laying female birds (Bloom et al 1941).

Lamellar bone is a more highly organised material and is formed slowly with parallel layers or lamellae comprising of an anisotropic matrix of mineral crystals and collagen fibres. Collagen fibres exist in two very different ''plywood'' architecture forms. The first of these is the classical view of lamellar bone, where collagen fibres run parallel in each lamella and change direction by ninety degrees at the lamellar interface. So that as the lamella builds up, the layers of collagen are put down in one orientation, then when the next lamellar layer is started, the collagen fibres are laid down at right angles to the previous layer (Giraud-Guille 1988) (Fig 1.3).

As a result of this, lamellar bone has certain properties, in that mechanical behaviour differs depending on the orientation of the applied forces. Moreover, its ability to resist loads is greatest when the forces are applied in parallel to the longitudinal axis of the collagen fibres. The second view is when the collagen fibres continuously change direction so that no individual lamellae are seen. The bone still shows lamellar structure as the collagen fibres rotate through one-hundred and eighty degrees. Both lamellae types of architecture are found in human cortical bone, but

distribution and interspecies variation have not been determined (Giraud-Guille 1988). Both forms have birefringence, in that the fibrous structures interact with polarised light. Lamellae are formed at the periosteum (outside edge), endosteum (inside edge) of the bone and also forming osteons. Osteons are lamellae structures which incorporate Haversian canals (see section 1.3.3). Using polarised light, osteons have been categorised depending on the bright and dark lamellae (corresponding to their collagen fibres exhibiting mostly circumferential, alternating, or longitudinal orientation) (Ascenzi and Bonucci 1967) (Fig1.4).

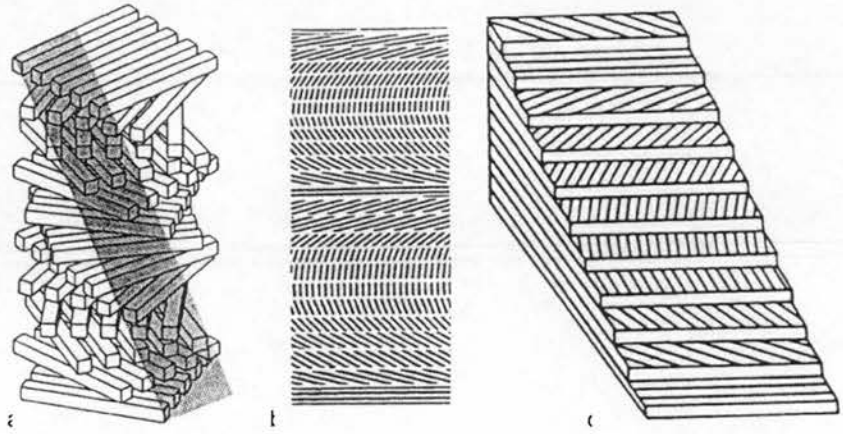


Figure 1.3 Diagram of helicoidal plywood structure. Three dimensional structure (a), effects of arches when an oblique section cut (b) as shaded plane in a, seen in an oblique section formed by helicoildal lamellae (c).
(Reproduced from Neville 1984)

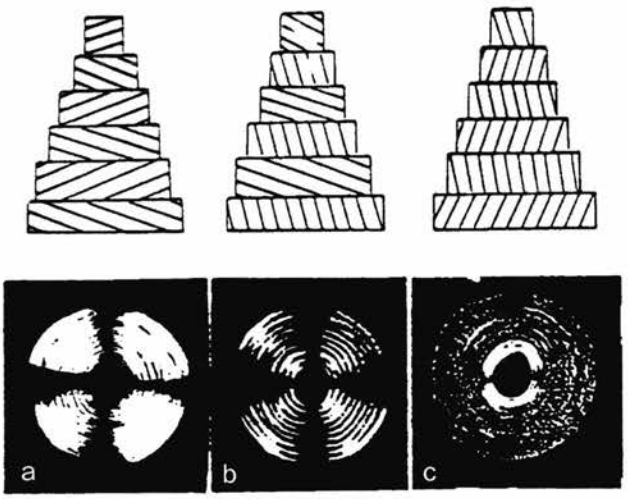


Figure 1.4 Three osteon types as defined Ascenzi and Bonucci. The top diagrams show hypothesized fibre arrangements and the bottom micrographs show appearance in polarised light. Transverse fibre orientation (a), alternating fibre orientation (b), longitudinal fiber orientation (c).
(Modified from Ascenzi and Bonucci 1967).

1.3.3: Bone Types Found in Long Bones

When a long bone is cut open transversly, two types of bone can be seen, one type of low porosity, cortical bone, and one type of high porosity, trabecular bone (Fig 1.5). Cortical bone is dense and forms a shell around the trabecular bone, with generally four times the mass of trabecular bone, but with a slower metabolic turnover rate. Cortical bone is composed of dense lamellae with three types of canals found within it. The first being Haversian canals named after Clopton Havers (1691). These are aligned with the long axis of the bone and contain capillaries and nerves, varying in cross-sectional shape from circular to oval.

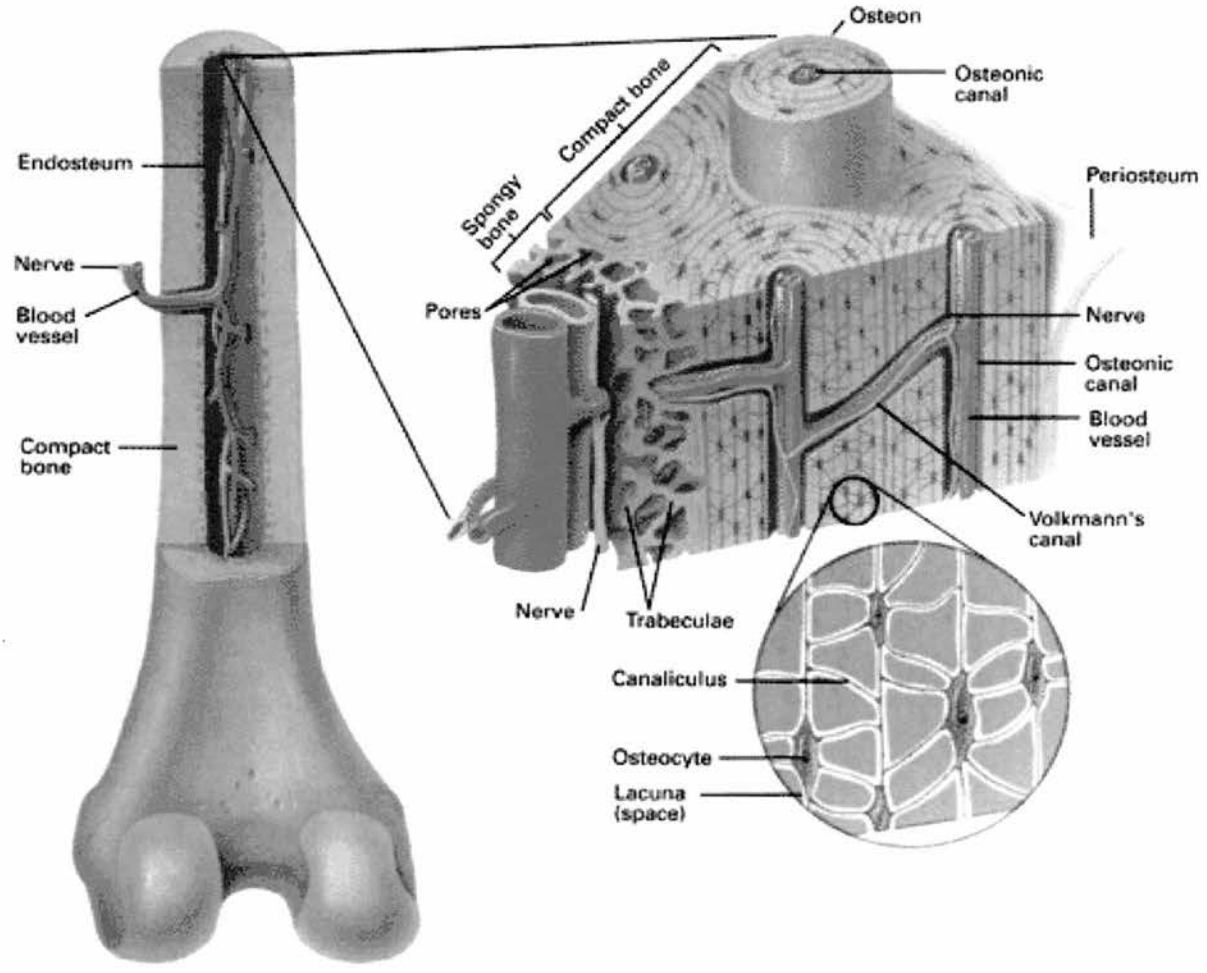
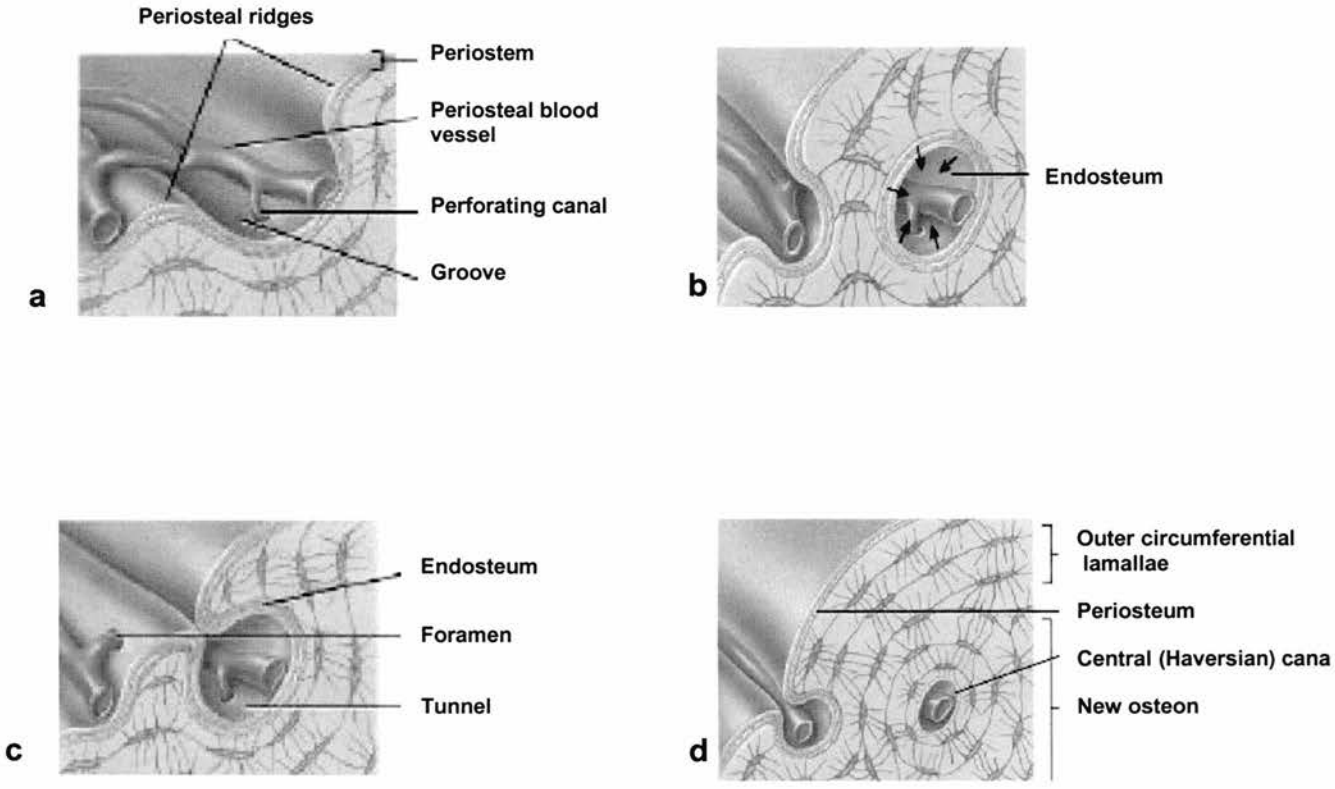


Figure 1.5 Micro structure of bone morphology

The Haversian network follows a gentle spiral around the axis of the bone in a clockwise direction on the left sided bones, and counter clockwise of right sided bones (Cohen et al 1958). Their length, calibre and density are strictly dependent on the nature of the bone they pass through, and on bone turnover processes, species and age (Marotti and Zallone 1980). The second type of canal are Volkmann's canals, named after Richard von Volkmann (1830-1889) which link Haversian canals

transversley and contain blood vessels and nerves (Cohen et al 1958, Martin et al 1998). The third type of canal are resorption cavities which are temporary spaces created by osteoclasts in the first stages of remodelling.

Cortical bone can be formed in several ways. Firstly as primary bone, where tissue is laid down on the periosteal or endosteal surfaces of existing cortices. There are two types of primary bone, circumferential lamellar bone, where the lamellae are laid down parallel to the bone surface. Primary osteons are formed within this by the incorporation of blood vessels from the periosteal surface. Longitudinal depressions form at the periosteal surface at the areas where blood vessels sit. These then become covered by more bone, so trapping the blood vessel within the bone. The resultant hollow cylinder contains blood vessels, nerves and osteoblasts which then form further rings of lamellae within the hollow, forming circular primary osteons. These are all part of the Haversian system, adding strength to the bone, and the formation of these structures demonstrate the continuity of the envelopes and establish that the origin of the osteonal endosteum, is originally from the periosteum (Banks 1986) (Fig 1.6). Plexiform bone is also primary bone, in which the rate of formation is greatly increased by continual construction of a trabecular framework on the periosteal surface with subsequent in-filling of the gaps between the trabeculae. This gives a brickwork appearance. This leaves a structure which is a mixture of woven and lamellar bone, and is viewed as a ''brick wall'' appearance. This is typical of fast growing animals such as cows, and has also been seen in racehorses that have been put under huge stresses and require more fatigue resistance (Stover et al 1992). However plexiform bone has not been seen in chickens even though they are a much faster growing species. This may be due to the differences between mammals and avian species.



a. A longitudinal depression/groove developes at the periosteal surface.
b. Differential osteoblast activity results in the groove becoming covered in bone.
c. Blood vessels and periosteal elements become encased in a tunnel.
d. Appositional bone growth within the tunnel results in formation of a primary osteon.

Figure 1.6 A diagram of primary osteon formation around a blood vessel at the periosteal surface.

Cortical bone can also be formed as a result of resorption of existing bone, with the replacement by new lamellar bone, this is know as secondary bone and the process known as remodelling. A prerequisite for secondary bone formation is the existence of a tunnel within the cortical bone. Using the primary osteon tunnels, the removal of the bone from the inner surface by osteoclasts, results in the expansion of the original limits of the primary osteon. The limit of expansion then becomes defined by the cement line/reversal line. This line defines the point at which resorption is finished and new bone is deposited by the osteoblasts (Banks 1986). Secondary osteons can be seen joining, overlapping or sitting isolated within the circumferential bone.

Trabecular bone is like a climbing frame of internal beams/plates of bone which is orientated along lines of stress. It is found in the marrow cavity and is under complex stresses and strains and is best designed for the resistance of compressive loads. It is composed of fine lamellae bone, and remodelling takes place on the surface at a much more active rate than that of cortical bone (Vaughan 1981).

1.4: Cellular Complement of Bone

Bone metabolism is regulated by bone cells which respond to various environmental signals, including chemical, mechanical, electrical and magnetic stimulus, as well as endocrine, including local and systemic factors. Specific responses are governed by cellular receptors found intracellularly or on the membranes of the cells. Cell membrane receptors bind the exogenous signal and transfer the information across the cell cytoplasm to the cell nucleus. Intracellular receptors bind the stimulus which has entered the cell and translocate the effector to the nucleus where it binds to a specific DNA promoter sequence of a gene. Not including the growth plate chondrocytes, there are four cell types found in bone; osteoblasts, osteoclasts, lining

cells and osteocytes. The first three are found on bone surfaces, whereas osteocytes are found within the mineral matrix. Bone formation takes place during embryonic development and growth, but also throughout life as processes of normal bone remodelling. Therefore the requirement for renewal of bone through remodelling, by resorption and formation, requires the proliferation and differentiation of osteoprogenitor cells (Lian and Stein, 1996) (Fig 1.7), and the presence of these cells ensure a plentiful supply of stem cells, used in growth, remodelling and repair (Banks 1986).

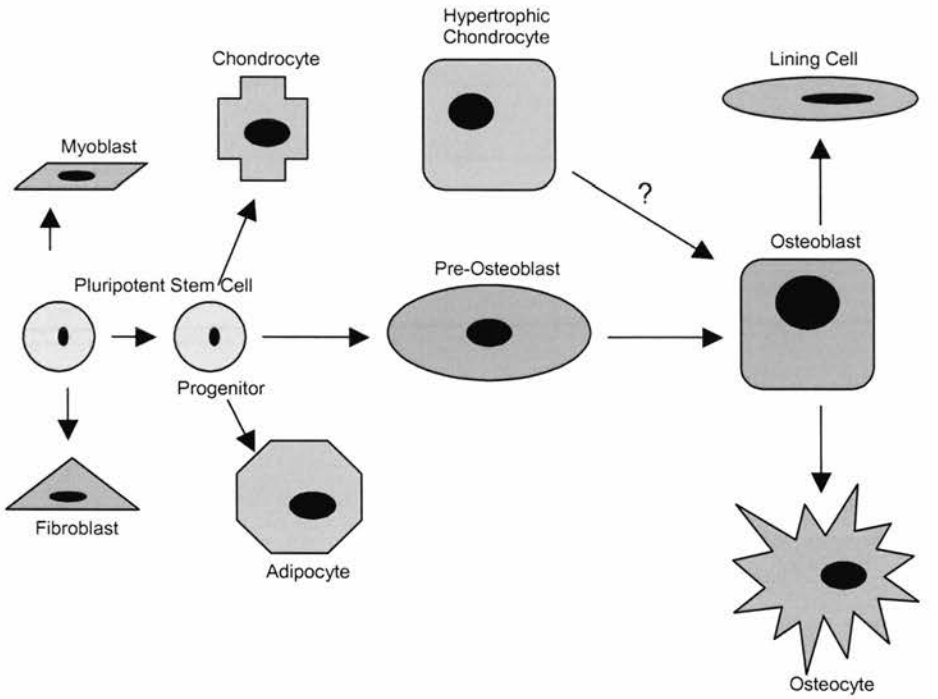


Figure 1.7 Diagrammatic scheme of a pluripotent stem cell and its pathway through maturation to mature cells.

1.4.1: Osteoprogenitors/Preosteoblasts

Osteoprogenitor cells originate from mesenchymal stem cells of the perichondrium (Friedenstein 1976, Owen 1978), with the earliest formation of bone in the perichondrium arising from cells that differentiate on the outer edge of the original mesenchymal condensation. Osteoprogenitor cells are committed to the osteoblast lineage, and are defined by their position relative to the bone tissue. They are generally observed one or two layers of cells behind the osteoblasts near the bone forming surface (Marks and Popoff 1988). These cells appear elongated but the closer to the osteoblasts they become, the more cuboidal like they appear. The main function of these cells, are to divide following mitogenic stimulation and differentiate into mature osteoblasts.

1.4.2: Osteoblasts

Osteoblasts are mononucleate cells which are characterised by the presence of membrane bound alkaline phosphatase (ALP), of which the amount present is dependent on the development state and functional activity of the cell (McLean and Urist 1968). Osteoblasts cover the bone surfaces in a monolayer of cells (Thomson and Loveridge 1992), and are specific to sites where apposition is taking place. They are columnar in shape, having originated from osteoprogenitor cells, which themselves are derived from the primitive mesenchyme of the perichondrium (Owen 1978). Osteoblasts are the bone forming cells, responsible for the formation of osteoid (Owen 1963), by secretion of procollagen and other non collagenous matrix components such as sialoprotein, osteonectin and osteopontin. They cover most bone surfaces, and have a weakly basophilic cytoplasm which surrounds a single nucleus, and during activity the cells hypertophy and become polarized (Banks 1986). Active

osteoblasts are 15-20 μm wide (McLean and Urist 1968) where the nucleus has a prominent nucleolus situated at the one side of the cell which is away from the bone surface. The cytoplasm contains a well developed Golgi apparatus and numerous mitochondria.

Osteoblasts are irregular in contour and have many fine cytoplasmic processes on the secreting side of the cell which extend into the osteoid matrix and contact with cellular processes of osteocytes (Cameron 1972, Ham and Cormack 1979). With established cell-cell communication with cells in the matrix, gap junctions are also often found between the osteoblasts on the surface as well (Yamaguchi et al 1994). Electron dense particles shown to have high calcium and phosphorous contents are occasionally found within the mitochondria. Blebbing from the plasma membrane of the osteoblasts are extracellular membrane bound particles of 100nm in diameter known as matrix vesicles. The micro-crystals of mineral formed during mineralisation are associated with these, and the inner aspect of the membrane (Anderson 1989, Ali 1992).

Osteoblast function is to synthesize and secrete a collagenous matrix/osteoid and then ultimately mineralise it (Owen 1963, Lian and Stein 1996). The osteoblast synthesises procollagen type I, which is excised and then cleaved to collagen extracellularly, whereupon it assembles into fibres (Prockop et al 1979). As well as synthesising pro-collagen, the osteoblast also secretes non-collagenous proteins such as osteocalcin, osteonectin and sialoprotein (Termine 1990). It is the collagen and non-collagenous protein plus proteoglycans (carbohydrate protein complexes), that make up the osteoid.

Mineralisation of the osteoid leads to the final stage of osteoblast differentiation, when the bone forming cells become incorporated into the matrix and differentiate

further into osteocytes or become quiescent on the mineralised surface designated as bone lining cells.

Cell growth and tissue specific genes have been mapped during the progress of the osteoblast through the differentiation pathway. In situ hybridization studies of osteoblasts in culture have identified four principal developmental periods (Stein and Lian 1995). Firstly; proliferation supports expansion of the osteoblast cell population to form a multilayered cellular nodule and biosynthesis of collagen type I. At this stage genes required for activation of proliferation are early response genes such as c-myc, c-fos and c-jun and cell cycle progression genes such are histones and cyclins (Smith et al 1995). These are expressed together with growth factors such as fibroblast growth factor (FGF) and insulin-like growth factor -1 (IGF-1) (Birnbaum and Wiren 1994) and cell adhesion proteins, fibronectin, and collagen type I (Stein and Lian 1995). Second stage; expression of genes associated with the maturation and organisation of the bone extracellular matrix are upregulated making the matrix competent for mineralisation. This is exemplified by the expression of ALP, with collagen synthesis continuing and undergoing cross-link maturation (Gerstenfeld et al 1993). The third stage involves gene expression related to the accumulation of hydroxyapatite in the extracellular matrix. These are the genes which encode proteins with mineral binding properties such as osteopontin, osteocalcin, and bone sialoprotein. These proteins exhibit peak expressions at the time of mineralisation. The fourth stage occurs in mature cultures where collagenase expression is elevated and apoptotic activity occurs. Compensatory proliferation activity and collagen expression is also evident (Lynch et al 1994).

The apparent lack of osteoblast specific markers and morphological features explains why the study of the transcriptional control of osteoblast differentiation has been

rather difficult in the past (Schinke and Karsenty 2002). With the identification of core binding factor 1 (Cbfa 1/ Runx2) (will be called Runx2 in this study) as a lineage specific transcriptional activator of osteoblast differentiation more insights have been recognised in osteoblast differentiation. Runx2 was originally cloned by Ogawa et al in 1993, and it's importance as a transcriptional factor of osteoblast differentiation was found four years later by several investigators (Ducy et al 1997, Otto et al 1997). Runx2 is a transcriptional factor belonging to the Runt family, containing a DNA binding region of 128 amino acids, which is known as the runt domain. This is followed C-terminally by a proline-serine-threonine rich domain, known as the PST domain, which contributes to the transactivation function of Runx2 (Aronson et al 1997). Compared to other Runt proteins Runx2 has two unique domains located at the N terminus that are also involved in activatating transcription. One of these is a QA-domain which is rich in glutamine and alanine, preventing heterodimerisation with Cbfβ, a known partner of other Runt family transcription factors (Thitunavukkarasu et al 1998).). Runx2 is expressed in cells of the osteoblast lineage and also chondrocytes but not in any other cell type or tissue (Ducy et al 1997). It is the earliest most specific marker of osteogenesis known to date. Confirming the importance of Runx2 as an osteoblast differentiation marker, knock out mice lacking the Runx2 gene died shortly after birth. These mice died due to defective endochondral and intramembranous bone formation, with complete maturational arrest in osteoblast differentiation, confirmed by the absence of osteoblast markers such as osteocalcin and osteopontin (Komori et al 1997).

1.4.3: Osteocytes

Osteocytes are considered the most mature or terminally differientiated cell of the osteoblast lineage. They are osteoblasts which have been embedded in their own secretory products, occupying spaces, lacunae, in the interior of the bone. Osteocytes are connected to adjacent osteocytes by cytoplasmic projections that are within channels known as canaliculi. The cell processes are long, rich in microfilaments and maintain contact with cell processes from other osteocytes or with those processes from the osteoblasts sitting immediately on the cell surface (Lian and Stein 1996). Osteocytes are responsible for the maintenance of the bone matrix and have the ability to synthesize and resorb matrix to a limited extent, using the projections as a sensory array to detect the need for bone remodelling (Lanyon 1992). Matrix producing osteocytes have cellular organelles similar to that of osteoblasts; where as osteolytic osteocytes contain lysosomal vacuoles and other features typical of phagocytic cells (Buckwalter et al 1996). The ability of these cells to form or resorb bone matrix is an important mechanism in the homeostatic maintenance of blood calcium levels (Banks 1986). Molecular strain plays an important part in the modelling and remodelling of bone, and the best situated cell to detect the strain is the osteocyte (Lanyon 1993). As the mechanical pressure displaces the extracellular fluid the surrounding matrix, including the osteocytes, are distorted (Turner et al 1994). The osteocytes react immediately to this, and the increased expression of other enzymes such as glucose-6-phosphate dehydrogenase appears rapidly (Skerry et al 1989). An increase in nitric oxide (NO) is also seen in response to mechanical loading, (Pistsillides et al 1995), which is generated by nitric oxide synthase (NOS). There are three types of NOS, firstly: neuronal, type 1 (nNOS), secondly: inducible, type 2 (iNOS), and thirdly: endothelial, type 3 (eNOS) (Bredt and Synder 1990, Pollock et al 1991, Xie et al 1992). The principle isoforms found in bone are eNOS

and iNOS (Fox and Chow 1998, Macpherson et al 1999), although nNOS mRNA has been shown in bone tissue (Pitsillides et al 1995). eNOS is the predominant isoform expressed by bone, and is present in osteoblast, osteocytes and osteoclasts (Fox and Chow 1998, Macpherson et al 1999). NO is involved in many aspects of bone growth both in modelling and remodelling under physiological and pathophysiological conditions. Osteocytes and osteoblasts produce NO when subjected to mechanical strain which has been shown to be dependent on the expression and activation of eNOS (Zaman et al 1999). Osteocyte apoptosis (cell death) is also considered a mechanism for site-specific targeted bone remodelling (Noble and Reeve 2000). By overloading ulnas of anaesthetised rats to the point of plastic deformation, large increases in apoptotic osteocytes were found before intracortical bone remodelling begun (Noble et al 1998).

1.4.4: Bone lining cells

These cells are osteoblasts that have escaped being buried in the bone matrix and remained on the surface of bone when bone formation has ceased. As production of bone matrix ceases the lining cells become quiescent and flatten out on the bone surface (Kimmel and Jee 1977, 1978) but do not form a continuous barrier over the bone. They still maintain communication with osteocytes and have gap-junctions between each cell (Martin et al 1998). Similar to osteocytes, they are thought to transfer minerals into and out of the bone, and sense mechanical strain (Parfitt 1987). With fewer organelles than an active osteoblast these cells are considered largely inactive (Miller and Jee 1987) but can be activated, by response to chemical and mechanical signals, to initiate bone remodelling (Miller and Jee 1992).

1.4.5: Osteoclasts

Osteoclasts are derived from the macrophage line, and are formed by the fusion of monocytes, originating in the hemopoietic portion of the bone marrow. They are multinucleated giant cells with their main function to degrade/resorb hard tissue upon contact with the bone matrix. The average number of nuclei is species dependent and also varies considerably within the individual (Kaye 1984). They contain multiple Golgi apparatus, a high density of mitochondria and a large number of lysosomal vesicles. The lysosomal vesicles originate from the Golgi apparatus and cluster at the ruffled side of the cell. The ruffled side is highly folded plasma membrane where bone resorption takes place. Releasing organic acids, citrate and lactate, the effect is to lower the pH of the immediate microenvironment suitable for osteolysis (Vaes 1988). On either side of the ruffled zone, is a smooth zone which is micro-filament rich and organelle free. This area is the point of attachment of the osteoclast to the underlying bone, with the osteoclasts adhering to the matrix through integrins (Parfitt 1984). This results in the formation of a tight seal, confining the pH change to the area of the ruffled border. The acids dissolve the bone mineral and enhance the activity of lysosomal enzymes that are released. Removal of apatite crystals results in a temporary layer of demineralised matrix below the ruffled edge of the osteoclast (Parfitt 1984). This is followed by digestion of the organic matrix by lysosomal enzymes such as tartrate resistant acid phosphatase (TRAP) which is transferred from the ruffled border to the sub osteoclastic space. Cysteine proteases, of which there are three, are able to both depolymerise fibrillar collagen and to degrade the resulting softened substance. These proteases are regulated by the differing calcium levels that exist in the ruffled border. Both phases of bone resorption are tightly regulated, and changes in osteoclast acidity result from various enzyme mechanisms within the

osteoclast: these include H^+/K^+ ATPases, Na^+/H^+ antiports (Baron et al 1985, Hall and Chambers 1990), chloride/bicarbonate exchanges, and carbonic anhydrase (Hall and Kenny 1985).

Cells of the mononuclear phagocytic system such as monocytes and tissue macrophages seem to be likely candidates for osteoclast precursors as they share a number of functional, cytochemical and morphological features with osteoclasts. It is however, still unclear the nature of the circulating mononuclear precursor. More recently investigations have shown that a bone derived stromal cell population is essential for osteoclast differentiation (Udagawa et al 1990), where it is now thought that these cells produce macrophage colony stimulating factor-1 (MCSF-1), which attaches to receptors on the precursor cells, so stimulating their differentiation into osteoclasts (Mundy 1999).

Receptor activator of nuclear factor-kappa β ligand (RANKL) is the same protein as that named osteoclast differentiation factor (ODF), and osteoprotegrin ligand (OPGL). Osteoclast differentiation and function are regulated by RANKL and MCSF-1. Osteoclast progenitors and mature osteoclasts express the receptor activator of nuclear factor-kappa β (RANK), which is the receptor for RANKL. RANKL is expressed by osteoblast and stromal cells, with expression stimulated by osteotropic factors such as parathyroid hormone (PTH) and vitamin D (Tsukii et al 1998). The up regulation of RANKL induces osteoclast differentiation, stimulating fusion and activation of the osteoclasts. However osteoblasts also produce osteoprotegrin (OPG), which acts as a decoy receptor of RANKL. By binding to RANKL, it prevents activation and fusion of the osteoclasts (Simonet et al 1997). With OPG acting as an important negative regulator of osteoclast differentiation and activation it ensures a balance between bone formation and resorption.

1.5: Control of bone formation and resorption

Bone formation and resorption is continuous through out life and a tightly regulated balance between the two mechanisms is necessary for the preservation of the skeletal mass and architecture. Therefore both of these processes are regulated through the actions of circulating hormones and a number of local control factors, some of which are influenced by mechanical loading/stress of the skeleton.

1.5.1: Hormonal and growth factor regulation

1.5.1.1: Parathyroid Hormone (PTH)

PTH is synthesised in the parathyroid glands as a mature protein. The parathyroid is very sensitive to circulating calcium (Ca^{2+}) levels, and when a fall in Ca^{2+} is detected by the recently cloned calcium sensor (a G-protein linked cell surface protein) (Brown et al 1993) PTH release is triggered. PTH promotes bone resorption, but it does not do this directly, but does so by first acting on cells from the osteoblast lineage which have PTH receptors (Silve et al 1982). By action on the osteoblasts through activation of cyclic AMP-dependent protein kinase, a number of post-receptor events follow (Livesey et al 1982). PTH increases the number and activity of osteoclasts in bone and the proportion of osteoclasts that show a ruffled border are increased as is the number of nuclei per osteoclast (Miller et al 1976).

1.5.1.2: Vitamin D

The active metabolite of vitamin D is 1α,25-dihydroxyvitamin D ($1,25(OH)_2D_3$) promotes the differentiation of the osteoblast and growth plate chondrocyte. The effects of $1,25(OH)_2D_3$ on bone formation and resorption are central to the hormones role in calcium homeostasis. (Lian and Stein 1996). $1,25(OH)_2D_3$ effects on bone are

often in conjunction with PTH, and result in the stimulation of differentiation (Owen et al 1991). By inducing the differentiatiated phenotype, the osteoblast progresses from an immature proliferating cell to a differentiated non dividing cell with the ability to synthesise matrix proteins and mineralised bone. Gene products regulated by $1,25(OH)_2D_3$ are osteocalcin, osteopontin and ALP (Haussler et al 1970, Price and Baukol 1980). It is also known that $1,25(OH)_2D_3$ stimulates bone resorption by directly influencing precursor cells to differentiate into mature osteoclasts (Bar-Shavit et al 1983). The process of osteoclastogenesis involves a complex interaction of osteoclast precursor cells, osteoblasts and bone marrow stromal cells. During the later stages of differentiation the osteoclasts loose their ability to respond to $1,25(OH)_2D_3$ and it is direct effects through the osteoblast lineage that induce and regulate osteoclast differentiation (Suda et al 1992).

1.5.1.3: Calcitonin (CT)

Calcitonin (CT) is a 32 amino acid peptide and the human and avian peptide differ by three amino acids, (Martin et al 1996). Avian CT's are the product of the ultimobranchial glands whereas in humans they are the product of the thyroid gland. CT acts directly on osteoclasts which contain a large number of receptors for the hormone. It affects the motility, shape and activity of the mature osteoclast and ultimately inhibits osteoclast activity and bone resorption (Martin et al 1996).

1.5.1.4: Estrogens

The principle circulating sex steroids in females are estradiol and progesterone which also function in males. The receptors for estrogen and progesterone in bone cells are low when compared to reproductive tissue suggesting a more limited range of actions

(Rickard et al 2002). Two estradiol isoform receptors are found in bone (ERα and ERβ) (Eriksen et al 1988, Komm et al 1988). Evidence indicates that the expression of both isoforms increases during osteoblastic differentiation *in vitro*, with the level of ERα mRNA initially low in proliferating cells but increasing at the onset of ALP expression, before reaching a maximum level in the fully differentiated osteoblast (Bodine et al 1998). When ERβ was studied in rat calvaria cultures, the expression levels were found to be maintained at a high level throughout the osteoblast differentiation cascade (Onoe et al 1997). It has been reported that the levels in rats of ERα and ERβ mRNA are lower in cortical bone than in trabecular bone with ERα being the predominant isoform in both bone types (Onoe et al 1997, Lim et al 1999). The expression of ERα mRNA has been isolated from chicken osteoclasts but ERβ has not been found in osteoclasts or their precursors (Ousler et al 1991).

1.5.2: Skeletal Growth Factors and Cytokines

1.5.2.1: Insulin-like Growth Factors.

Insulin-like growth factors (IGF's: IGF-I, IGF-II) are 7kDa polypeptides, found in high concentrations in serum. The skeleton is a major source of IGF-I and IGF-II through synthesis by bone cells and their release from the matrix during active skeletal resorption. Generally the relative proportion of IGF-I: IGF-II is maintained in both serum and the skeleton of various species (Bautista et al 1990). PTH and other inducers of cyclic AMP in bone cells appear to be primary stimulators of IGF-I synthesis in osteoblasts (Raisz et al 1993). IGF-I and IGF-II have similar effects on bone formation but IGF-I is more potent than IGF-II (Canalis 1980). IGF's are modest mitogens for bone cells, increasing the replication of preosteoblastic cells which ultimately differentiate into mature osteoblasts. They also enhance collagen

type I synthesis and promote bone matrix apposition rates thereby increasing bone formation (Hock et al 1988).

1.5.2.2: Interleukin-1

Interleukin-1 (IL-1) is a multifunctional cytokine with a wide variety of activities. It has been shown to regulate bone resorption, (Lorenzo et al 1987). IL-1 is the most potent stimulator of bone resorption identified. It increases prostaglandin synthesis in bone (Lorenzo et al 1987) which themselves are resorption stimuli (Klein and Raisz 1970). In the presence of iNOS, both *in vivo* and *in vitro*, IL-1 stimulates bone resorption (Van't Hof et al 2000), and similar to other resorption stimuli it increases receptor activator of RANKL production in stromal and osteoblastic cells (Hofbauer et al 2001).

1.5.2.3: Tumour Necrosis Factors

Tumour necrosis factor (TNF-α and TNF-β) have similar biological activities as IL-1. They stimulate bone resorption (Lorenzo et al 1987) and also inhibit collagen synthesis (Canalis 1987). Resorption of bone is due to an increase in osteoclast number which is mediated by increases in RANKL expression, in osteoblastic cell models (Hofbauer et al 1998, 1999). TNF directly inhibits the differentiation of osteoblast precursor cells into mature osteoblasts (Gilbert et al 2000), which involves the production of NO and peroxynitrite. Apoptosis (cell death) of osteoblasts is stimulated by TNF and this response can by mediated by NO production (Damoulis and Hauscka 1997).

1.5.3: Neural Control of Bone

Osteocytes are believed to act as censor cells that translate mechanical stimuli resulting from the gravitational and muscular forces on the skeleton into biochemical signals. These biochemical signals in turn activate the effectors of bone turnover: osteoblasts and osteoclasts, leading to adaptation of mass and structure of bone (Chapter 1, section 1.6.1). Activities of osteoblasts, osteoclasts and osteocytes, such as proliferation and differentiation of their precursors are regulated by a multitude of factors. These factors are humoral (hormones), local factors (growth factors and cytokines), and neurotransmitters such as neuropeptides (Lerner 1996). Recently the presence of another regulatory system acting on bone has been found, primarily known as a neural system.

1.5.3.1: Serotonin receptor

One of the genes found to be preferentially expressed (not exclusively) by osteocytes was the serotonin receptor (5-hydroxytryptamine (5-HT) receptors 5-HT_{2A} , 5-HT_{2B} , 5-HT_{2C}), sub type 5-HT_{2B} (Westbroek et al 2001). The serotonin receptor family is split into seven subfamilies (5-HT $_{1\text{-}7}$ receptors), each further divided into a number of subtypes ($_{A}$, $_{B}$ and $_{C}$). Except for 5-HT $_{3}$ receptor, serotonin receptors belong to the G-protein-coupled receptor superfamily which is characterized by seven membrane spanning hydrophobic regions, with an N-terminal extracellular and a C-terminal intracellular domain (Hoyer et al 1994). The Serotonin receptor 5-HT $_{2B}$ mRNA is expressed in various tissues, in part due to the presence of the receptor in endothelial tissue (Ullmer et al 1995, Ellis et al 1996), although this particular receptor subtype has low expression in the central nervous system compared to other receptor subtypes (Duxon et al 1997). The one group that has examined the expression of 5-HT $_{2B}$ in bone from chickens concluded that the high degree of 5-HT

$_{2B}$ receptor mRNA in osteocytes was related to their function as mechanosensors. 5-HT $_{2B}$ receptor was also found to modulate the response of osteoblasts when subjected to pulsating fluid flow (Westbroek et al 2001).

1.5.3.2: Glutamate receptors

Functional glutamate signalling has been found in non-neuronal tissues such as bone, pancreas and skin (Skerry and Genever 2001). These findings raise the possibility that glutamate acts as a wide spread "cytokine", being able to influence cellular activity. Glutamate was originally studied as a neurotransmitter (Hollmann and Heinermann 1994). Expression in rat bone of glutamate/aspartate transporter (GLAST) was down regulated with loading *in vivo* (Patton et al 1998). Further studies *in vitro* confirmed GLAST protein expression was mechanically regulated in osteocytes and osteoblasts (Mason et al (1997). Glutamate receptors are divided into two groups, 1) G protein-coupled metabotropic receptors and 2) ionotropic glutamate gated ion channels [N-methyl-D-aspartate (NMBA) and kainite receptors]. The NMDA receptor has been found in bone (Patton et al 1998).

1.6: Bone modelling and remodelling

As long bones develop the shafts grow in diameter as well as length. Growth in diameter is through periosteal intramembranous ossification. However the need to shape the bone to accommodate the growing/changing weight and mass of the animal is necessary. Therefore bone must be removed in some places and added to in others. This is known as modelling. Osteoclasts resorb bone and osteoblasts form bone, with both cell types working at different areas. The difference to bone remodelling is that

osteoblast and osteoclasts work together in a coupled action at the same site to remove and replace bone (Martin et al 1998).

1.6.1: Modelling

Modelling becomes necessary as the bone lengthens. When it is laid down originally it is not of the correct geometry. Therefore it needs to be customised to each particular animal due to each using their skeleton in subtle but different ways. To customise, the bone modelling must take place at the metaphyseal and diaphyseal areas. As a long bone grows the diameter of the cortical bone created at the metaphyseal area, below the growth plate, needs to be reduced to create the diaphysis region. This is carried out by osteoclasts on the periosteal surface of the metaphysis reducing the shaft to the required size. As an animal grows the long bones increase in diameter by action of the osteoblasts in the diaphyseal area, through slow periosteal intramembranous ossification. Simultaneously, cortical bone is removed at the endosteal surface by osteoclasts, thereby increasing the medullary cavity. Curvature of long bones, known as diaphyseal drift, is also adjusted as the animal grows to accommodate mechanical loading (Martin et al 1998) (Fig1.8).

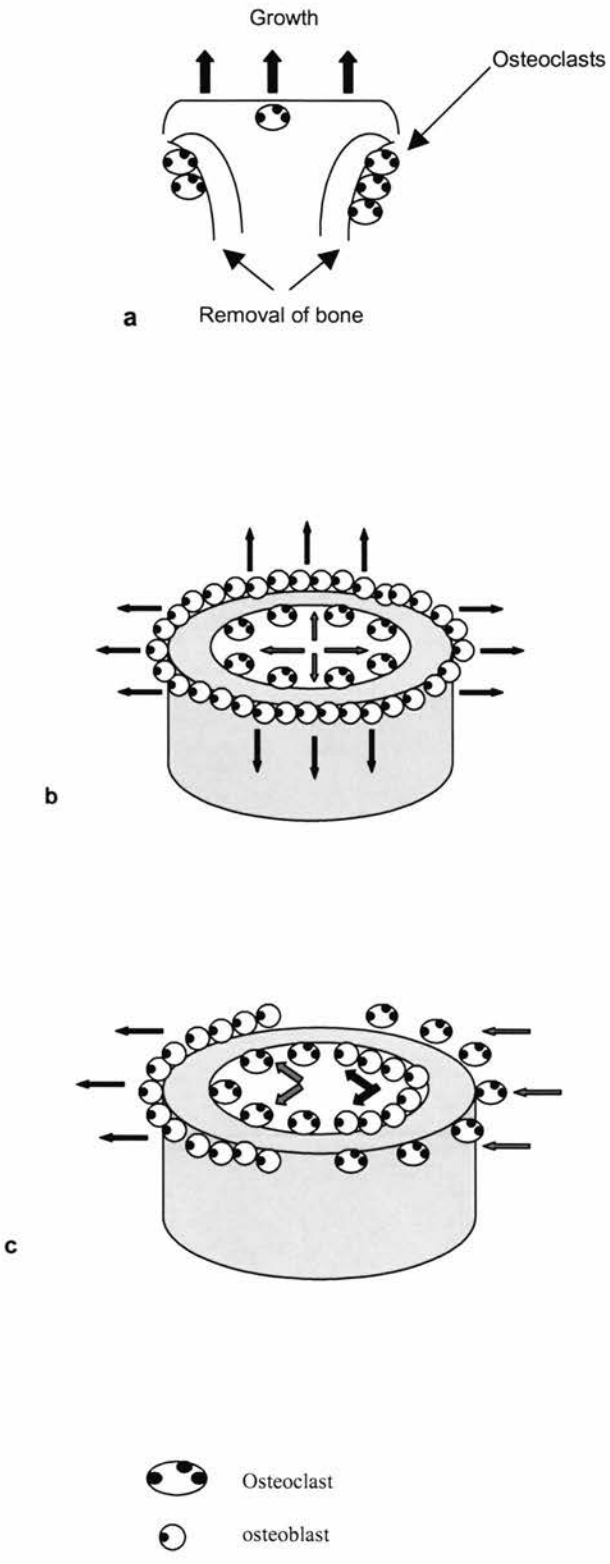


Figure 1.8 Diagram of bone modelling, with metaphyseal cut back (a), diaphyseal enlargement (b) and diaphyseal drift (c).

1.6.2: Remodelling

This term is used where portions of older bone are replaced with new bone. There are no gross changes in bone mass or shape only subtle replacements (Frost 1973, Banks 1986). Remodelling occurs continually throughout life and can be seen by the varying amounts of mineralisation around the Haversian canals (Vaughan 1981). This process also repairs any microscopic damage and prevents the accumulation of fatigue damage that could lead to fractures, (increasing mechanical efficiency) (Hert et al 1972, Frost 1986, Martin et al 1998). It has been suggested that mechanical energy is transducted into electrical energy by the bone structure and vascular structures within, letting the bone know where, how and in what orientation to start remodelling (Vaughan 1981). However, various endocrine and mechanical signals, thought to be produced locally, can result in bone remodelling (Rawlinson et al 1991). Increased bone cell metabolism, shown by an elevated glucose-6-phosphate dehydrogenase activity is followed by elevated RNA production and this was also found to be a trigger for bone remodelling (Pead et al 1988). Continual mechanical stress can cause micro fractures that must be repaired. Mechanical stress can also necessitate the repositioning or orientation of osteons to accommodate the tension and compression placed on the bone at specific areas. Although a vascular supply is incorporated when ostoens are formed, sometimes the vascular supply moves too far away from the osteocytes causing them to die, necessitating remodelling to minimise osteocyte death (Banks 1986, Noble et al 1997). Finally, changes in endocrine signals can also cause remodelling to take place.

Remodelling occurs by the combination of both osteoclasts and osteoblasts working together in a basic multicellular unit known as a BMU. This generally has on average

ten osteoclasts and several hundred osteoblasts where three principle stages are seen in a BMU lifespan, activation, resorption and formation (ARF) (Fig1.9).

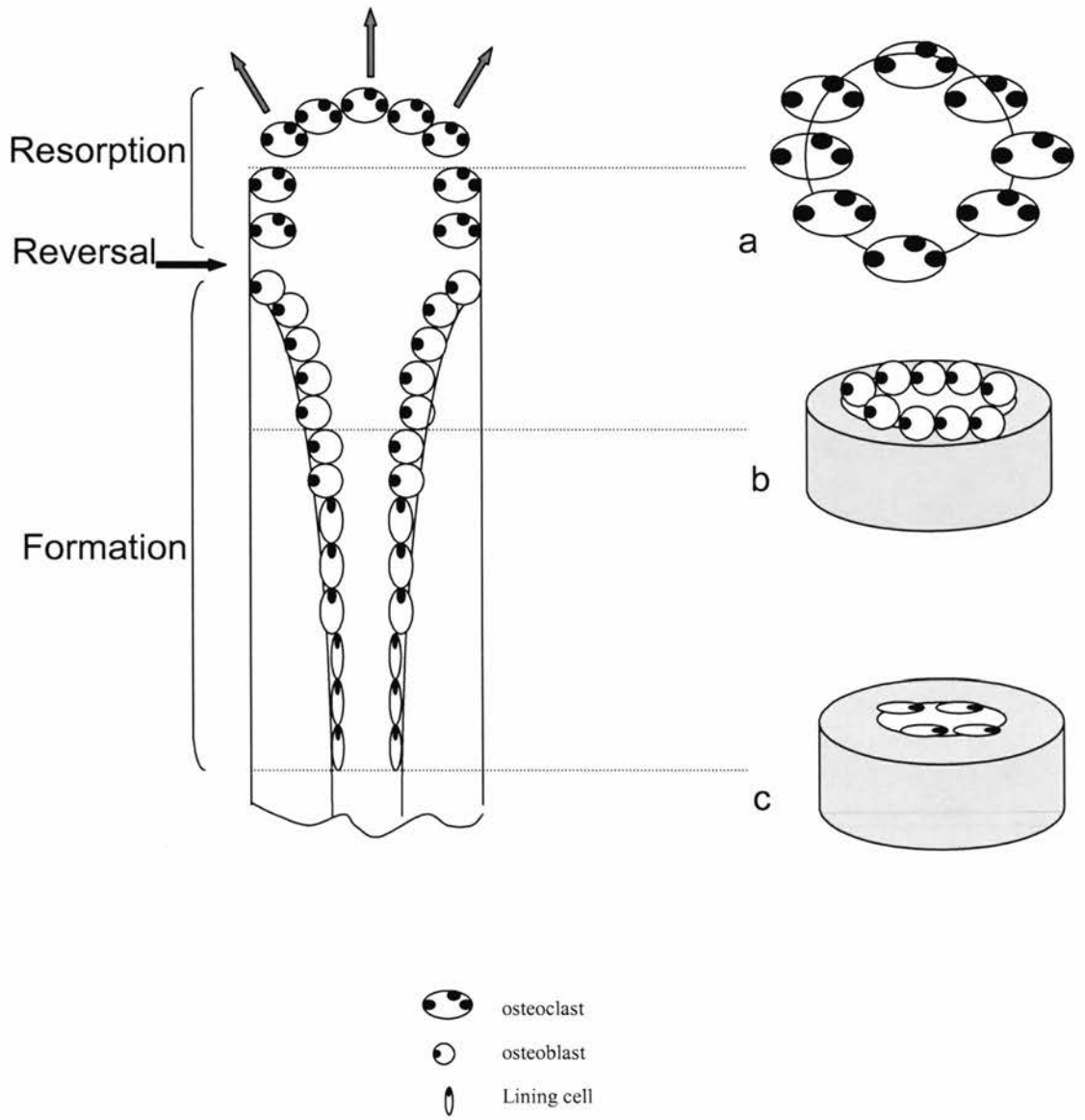


Figure 1.9 Diagram of a BMU, where osteoclasts at the head of the cutting cone resorbing bone (a), osteoblasts following the osteoclasts, laying down new bone (b) and mature osteon with lining cells in the Haversian canal (c).

The ARF process may be further broken down to six separate stages. 1); Activation of the unit occurs when the above described signals cause osteoclasts to form from monocytes. 2); Resorption is initiated when the osteoclasts begin the removal of bone at specific regions within the cortical bone. The osteoclasts form at the head of the cutting cone. Which contains both a capillary bud to supply nutrients and also progenitor cells for osteoclasts and osteoblasts. This cutting cone forms a tunnel that progresses through the cortical bone, and as a vascular system is always maintained, the tunnel never entirely closes, and thereby forms a new Haversian canal within a new secondary osteon. 3); Reversal of osteoclastic bone resorption to osteoblastic bone formation. The length of this region varies dependent on the lag time between the two activities. In a completed secondary osteon a cement line identifies the location of where reversal has occurred. 4); Bone formation occurs when osteoblasts formed from mesenchymal cells, begin to replace the resorbed bone by laying down concentric lamellae (Martin et al 1998). Formation is always much slower than resorption, with total remodelling taking about four months in the human. In man, BMU's are thought to replace about 10% of cortical bone each year. This rate is higher in children, reducing in young adults before increases and falls again in older individuals. The rise again in later years is related to the menopause in women (Frost 1964). 5); Mineralisation occurs of the organic matrix of the osteoid and mineral is deposited along the length of the collagen fibres. Once started about sixty percent of the mineralisation occurs during the first few days; primary mineralisation, and the remainder mineralised at a decreasing rate over the next six months; secondary mineralisation (Parfitt 1983). Finally, 6); The quiescence stage. When the tunnelling and infilling is completed, the osteoclasts disappear, and osteoblasts are either

incorporated into the matrix as osteocytes or become lining cells within the new Haversian tunnel.

Bone remodelling is not confined to intracortical bone but also occurs on trabecular bone surfaces. Trabecular bone remodelling is carried out on the surface of the bone, where the osteoclasts resorb bone, forming an indented groove known as Howship's lacunae. These areas then become filled in with newly formed osteoid laid down by osteoblasts and mineralisation occurs there after (Einhorn 1996) (Fig 1.10).

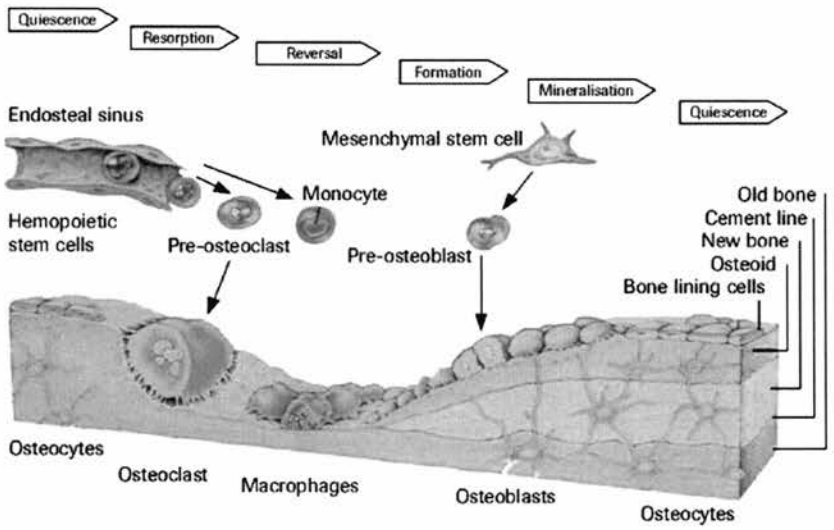


Figure1.10. The bone remodelling cycle showing the interplay between all the major celltypes(Imagefrom http://www.roche.com/pages/facets/11/bone_remodelling2.jpg).

1.7: Mechanical properties of bone

Mechanical properties of bone are governed by the same principles as that of man-made load bearing structures. Animals however have the ability to adapt their bone

structure to the loading imposed on them. The strength of a structure may be defined as the load at the failure point, where upon the stiffness of a structure is the load required to deform it a certain amount. This being the same as, what is known as, the load deformation curve.

Changes in mechanical function can produce corresponding changes in bone architecture (Wolff 1892). This relationship is commonly known as Wolff's Law. When considering a non-living object, loading can cause mechanical deformation either instantly, or if repeated often enough, fatigue failure. With bone, repeated deformations through normal mechanical function has a physiological influence, acting to maintain orientation and mass to suit the structural demands made on it (Lanyon and Bourn 1979). Stress is a measure of bone strength that considers differences in size and shape of the materials being tested.

Porosity in structural materials exerts strong influences on mechanical properties (Brown et al 1964). A similar phenomenon is observed in bone. Strength and stiffness vary inversely with increasing porosity, and conversely strength and stiffness improve as density increases (Martin 1984). Therefore with porosity increased, density, elasticity, strength and stiffness decrease. Mineralisation does not by itself directly influence stiffness. Quality refers to how completely bone is mineralised and to what extent the compact bone has undergone remodelling. Both factors will effect elasticity (Currey 1975). Within cortical bone the elastic modulus is highly influenced by the volume of bone present and the quality of the lamellar bone within (Schaffler and Burr 1988).

1.8: Blood supply of long bones

The blood supply to long bones comes from three sources, the nutrient artery, the periosteal arteries and the epiphyseal arteries. However it is more informative to describe long bone blood supply according to the anatomic location, that being, the afferent vascular system (arteries and arterioles, nutrient carrying), efferent vascular system (veins and venules, waste product removal) and intermediate vascular system (linking afferent and efferent by capillaries in cancellous bone and Haversian systems) (Rhinelander 1974). The periosteal arterioles make up part of the afferent vascular system, but only contribute a minor supply to the diaphyseal cortex. It supplies the external one third of the cortex (Rhinelander and Wilson 1982). The major afferent supply to the long bone is by the nutrient artery which enters the long bone traversing the full thickness of the cortex to enter the medulla. Once within the bone the branches subdivide into arteriole branches which both ascend and descend. These enter the endosteal surface of all portions of the diaphysis providing the afferent supply to the inner two thirds of the cortical bone. This is complemented by the metaphyseal arteries that anastomose with terminal branches of the nutrient artery within the medulla (Cooper and Cawley 1988). The periosteum, which covers the diaphyseal cortex, contains vascular connections.

1.8.1: Blood vessel composition

Blood vessels are comprised of three anatomical layers, where the intima (inside layer) which is composed of a monolayer of endothelial cells rests on a basement membrane. This basement membrane contains a unique type of collagen (type IV), associated with two structural glycoproteins, laminin and entactin, with the major proteoglycan being heparan sulphate (Campbell 1987). Cells rest on the lamina, transversed by fibrils which are laminin rich (Leivo and Wartiovaara 1982). The

functional characteristics of basement membranes depend on their molecular constituents, and it has been shown that the glycoproteins laminin (Timpl et al 1979, Leivo et al 1982) and fibronectin (Yamanda and Olden 1978, Ruoslahti 1981) have effects on cellular behaviour and interactions. Laminin appears present exclusively in the basement membrane, even at early stages of development (Leivo et al 1980, Wartiovaara et al 1980). Laminin is a large (850kD) glycoprotein, and is one of the first extracellular matrix proteins synthesised in a developing embryo. It is a flexible complex of three very long polypeptide chains arranged in the shape of an asymmetric cross and held together by disulphide bonds (Fig 1.11).

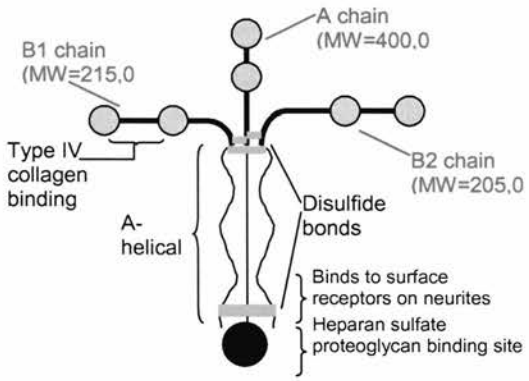


Figure 1.11 Diagram of laminin structure

1.9: Aims of Thesis

Although it is well accepted that bone architecture adapts to withstand the loads placed on it, the manner in which this occurs in the immature growing skeleton is not fully understood. Previous studies have used an avian model which compared two strains of birds: a modern fast growing strain undergoing selection for growth performance and skeletal health, and a slow growing control strain that has not been subject to selection since 1972 (Williams et al 2000a, Williams et al 2000b). These studies indicated that the fast growing strain had increased cortical porosity compared with the slow growing strain which may contribute to the observed skeletal failure in the modern broiler (meat-type) bird. Such modern birds can grow twice as fast as their unselected predecessors (Fig. 1.12) and in response to this increased load during growth, bones increase their circumference and increase their cross sectional moment of inertia by the rapid incorporation of primary osteons.

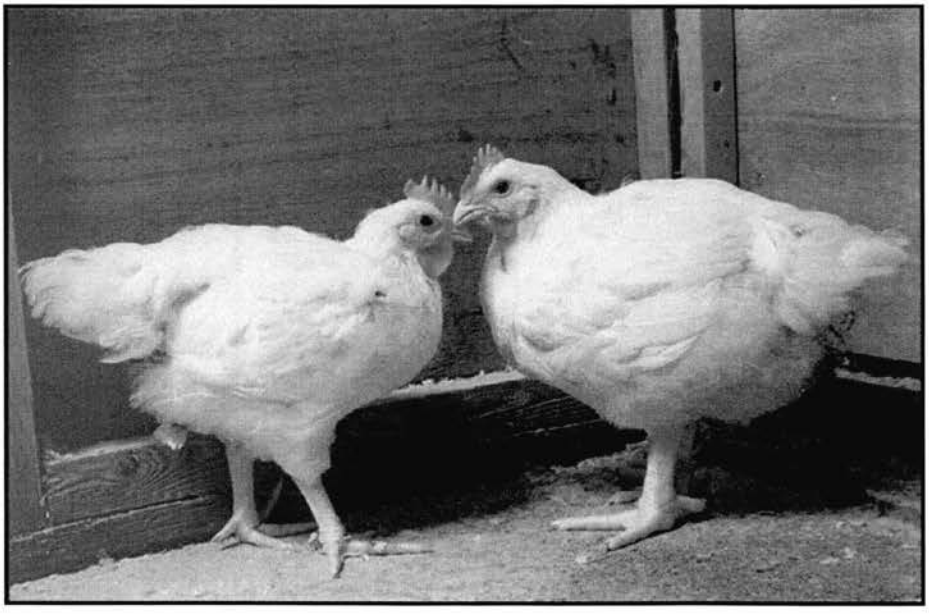


Figure 1.12 The slow growing control strain (left) with the fast growing broiler (right). Both birds are of 42 days of age. Note the size in diameter of the visible leg bones.

These initially form at the periosteal surface and osteoblasts then infill the resultant canal. However, if growth is too rapid then this process may fail so that while an increasing number of primary osteons are incorporated they fail to infill leaving a porous and weaker structure. The primary aims of this study were therefore to understand the effects of growth rate on cortical bone quality. In addition, the cellular and molecular mechanisms regulating osteonal formation and infilling were also studied in an attempt to determine the cause of the increased porosity associated with fast growth. The experiments described in this thesis use an interdisciplinary approach integrating cell and molecular biology with morphometric and mechanical assessment of bone.

This work has implications for all growing species where the long bones must adapt to the growing body. This is particularly relevant to the poultry industry, which is blighted by an extremely high incidence of bone fracture within its broiler flocks. A greater understanding of how bones respond to the ever increasing muscle mass may be important for the development of strategies to prevent bone failure during growth.

1:10 Hypothesis

The hypothesis to be tested is that rapid growth of the immature skeleton results in poor quality of cortical bone.

Chapter 2: Materials and Methods

2.1: Whole Animal *In Vivo* studies

2.1.1: Housing and Feeding of animals (1)

In study 1 (chapter 3) two strains of bird were used: a current fast growing strain, and a slow growing control strain that has not been subject to genetic selection since 1972. Twenty-one day-old male chickens (n=20) of the control and fast growing strain were placed into four brooders. The brooders were grouped together, with each brooder containing either ten of the fast growing strain or ten of the control strain. Lighting was set at 23 hours light, 1 hour dark for the duration of the experiment. All birds were fed *ad lib* on standard broiler diet and at the point of cull all chickens were weighed.

2.1.2: Housing and Feeding of animals (2)

In study 2 (chapter 4) two strains of bird were used: as described in (1). One day-old male chickens of the control and fast growing strain were placed into two floor pens, one pen for each strain. Lighting was set at 23 hours light, 1 hour dark for the duration of the experiment. All birds were fed *ad lib* on standard broiler diet and at the point of cull all chickens were weighed.

2.1.3: Tissue extraction (1)

At 14, 21 and 42 days of age birds were culled by cervical dislocation, weighed, and both tibiotarsi complete with periosteum, were removed. From the right bone, two transverse section of the diaphysis were cut (approximately 5 mm in length) using Mini Precision Drill (RS Components, Corby, UK.), fitted with a diamond-cutting wheel of 22 mm diameter (Fig 2.1). One section was frozen, and section two was

decalcified and wax embedded. Tibiotarsi were all orientated with the proximal end to the left, and the sections were cut from the central mid, area from the diaphysis. The left tibiotarsi were used for three point bending (chapter 3, section 3.3.2).

At the beginning of this study some archival cortical bone tissue was available at 14 and 42 days of age. On this basis the initial studies were carried out using the same age of tissue, however as results showed no difference between the two ages, a midpoint of 21 days of age was used when collecting tissue for further studies. At 21 days the chickens are still on a steep growth curve and therefore provide a good example of a rapidly growing immature skeleton.

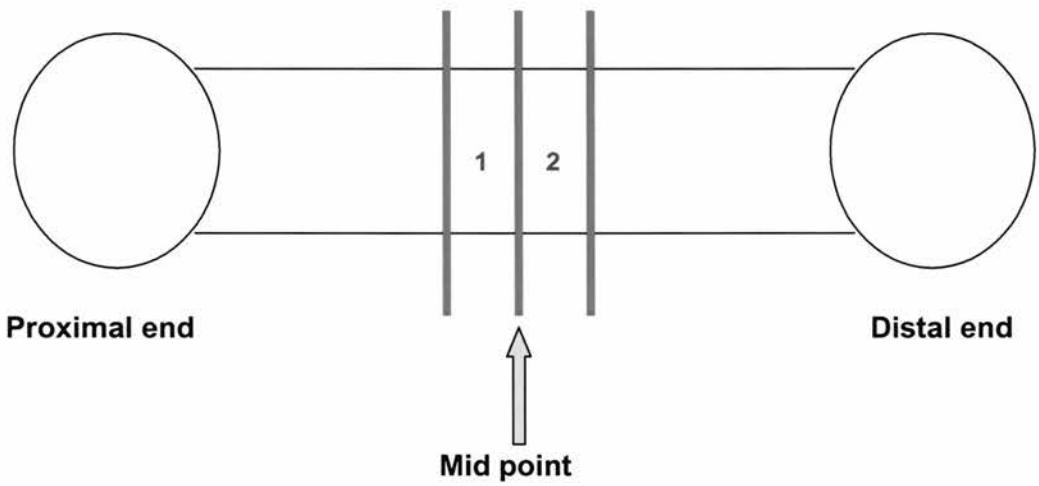


Figure 2.1 Diagram of area cut from dissected bones for BrdU, blood vessel, osteocyte density, primary osteon infilling and calcein studies.

2.1.4: Tissue extraction (2)

At 21 days of age, birds were culled by cervical dislocation, weighed, and both tibiotarsi complete with periosteum, were removed. Seven transverse sections of the diaphysis were cut (approximately 5 mm in diameter) as previously described (section 2.1.3). Tibiotarsi were all orientated with the proximal end to the left and sections were cut as follows. The top three sections (1-3) were placed into 70% alcohol for 24 hours and decalcified in 10% EDTA (pH 7.4) for two weeks at 4°C. Thereafter two of the sections (1-2) were processed into RNAse free paraffin blocks, using established protocols (section 2.1.5.1) and one frozen (section 2.1.5.2) using a hexane freezing bath to be used for LCM. The fourth diaphyseal section was directly frozen in the hexane bath after dipping in 5% polyvinyl alcohol (Sigma, Poole,UK) for possible use with LCM. The fifth section was placed in 4% paraformaldehyde (Sigma), and sixth and seventh in live/dead cell probes (Molecular Probes, Paisley,UK) for detection of apoptosis, for 24 hours, decalcified in 10% EDTA for two weeks at 4°C, and processed into paraffin blocks (section 2.1.5.1) (Fig 2.2). This protocol ensured adequate tissue was prepared for several methods of analysis from one bone and reduced the number of experimental chickens required.

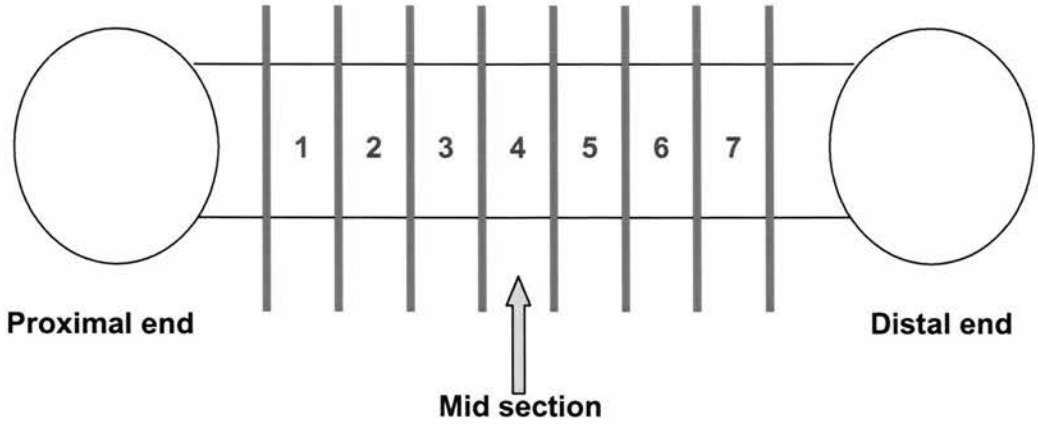


Figure 2.2 Diagram of cortical bone sections cut from dissected bones for laser capture microscopy (LCM), and live/dead studies.

2.1.5: Histological Processing

2.1.5.1: Wax sections

Fixation

At the time of dissection, pieces of bone tissue were trimmed to the required size, placed in labelled universals containing 70% alcohol (70% alcohol and 30% distilled water (dH_2O)). The tissue was left for 24 hours at room temperature for fixation.

Decalcification

After fixation the alcohol was removed and replaced with 10% EDTA (Sigma) (pH 7.4). The tissue was kept at 4°C for two weeks on a rotator, with repeated changes of EDTA (every second day). Samples were checked for total decalcification at the end of this period by x-raying at 35 kVp for 15 seconds (Faxitron 804, Livingston Electronics Ltd, Watford, UK). If any residual mineral was identified (seen as white

area on the x-ray) the samples were returned to the EDTA solution. If samples were clear, the tissue was washed in dH_2O then processed and embedded into wax blocks.

Sample Processing

Samples were processed by hand after decalcification. The tissue was removed from the EDTA in the universal and placed individually into labelled cassettes within a Duran bottle containing 70% alcohol. The following day the 70% alcohol was changed into fresh 70% alcohol and left for 30 minutes. This was then replaced with 80% alcohol and left for 30 minutes before being changed into fresh 80% alcohol for a further 30 minutes). This was then replaced with 95% alcohol, two changes of 30 minute durations (kept at 4°C throughout all alcohol stages). Finally the cassettes were left in 95% alcohol overnight at room temperature (RT).

The 95% alcohol was then changed for 100% alcohol and left for 2 hours with 1 change after 1 hour. A further change into xylene 2 x 1 hour was carried out, with the Duran bottle being placed on a roller in a fume hood. Cassettes were then placed into pre-melted wax for 2 x 1 hour periods.

Embedding and cutting

Once processed, the tissue was embedded in paraffin wax with a melting point of 60°C using appropriate sized plastic moulds. The wax blocks were allowed to cool, and excess wax was trimmed away in 15μm slices on the microtome to leave the sample surface exposed for cutting. Once trimmed, the blocks were cooled at -20°C for 30 minutes before sections of 5μm thickness were cut. The sections were transferred to a water bath which was at 40-50°C and left to soften for 1 minute before being transferred to a poly-l-lysine-coated microscope slide (VWR

International Ltd, Lutterworth, UK). The slides were then placed in an oven at 50°C overnight to ensure a good attachment of the sections to the slide.

RNAse Free Wax Embedded Tissue

All processes were carried out as above, but all solutions were made with diethyl pyrocarbonate (DEPC) (Sigma) water in RNAse zap (Ambion, Huntingdon, UK) treated glassware. All tools and the water bath used during the cutting of sections were similary treated with RNAse Zap. The water bath was filled with DEPC treated water and all blocks were handled with gloved hands. RNAse free and disposable knives were used throughout.

2.1.5.2: Frozen sections

(After Farquharson et al 1992).

Preparation of freezing bath

A large glass jar with lid was inserted into a polystyrene base, and filled 1/3 full with absolute alcohol. A small beaker was placed inside the jar and dry ice chips were added to the alcohol surrounding the beaker until a saturated solution was obtained (the mixture became viscous and stopped bubbling). Hexane was then poured into the beaker and the lid screwed on. This was left for 30 minutes to reach optimum temperature of -70°C.

Preparation of polyvinyl alcohol (PVA)

Polyvinyl alcohol (PVA) (Sigma) aids the cutting of frozen mineralised tissue. A 5% solution was prepared by gradually adding 5 g of PVA to 100 ml of warm water on a heated magnetic stirrer within an extraction hood. The solution was left stirring at a low heat for 1 hour, and then allowed to cool.

Freezing and Cutting of Tissue

The bone tissue samples were individually dipped in the PVA and then dropped into the hexane. After 30 seconds, they were retrieved using forceps pre-cooled in dry-ice, placed into pre-cooled self-sealing bags containing a piece of tissue to absorb any remaining hexane. The tissue was stored at -80°C until use. Using optimal cutting temperature compound (Brights, Huntingdon, UK) to attach the samples to metal chucks, sections of 10 μm were cut at -30°C (Brights, OT model cryostat), and picked up on poly-l-lysine coated microscope slides. The sections were then air dried at RT.

RNAse Free Frozen Tissue

All processes were carried out as above, but all solutions were made with DEPC treated water in RNAse zapped glass ware, as were the knife and all tools for handling the tissue samples. Gloves were worn when handling the tools and slides.

2.1.5.3: Demasking of wax embedded sections for optimal detection of basement membrane components by immunohistochemistry

Three methods were tried to unmask wax embedded bone sections for use with antibodies (section 2.1.6) and the two basement membrane antibodies which worked best (3H11 and 33) (section 2.1.7). All demasking agents were sourced through DAKO (Ely, UK) and used according to instructions provided.

1 Trypsin 3pk

Trypsin 3pk was reconstituted by combining the trypsin powder with the buffer supplied and brought to RT. 100 μl of the trypsin suspension was added to each section and incubated at 37°C for 20 minutes before washing

with PBS. The sections were then ready to continue with blocking and antibody staining.

2 Proteinase K (prediluted)

Proteinase K was bought ready to use and placed onto slides for 10 minutes at RT and then washed in PBS. The slides were then ready for blocking and antibody staining.

3 Pronase

This was made up into a working solution of 1%, from a 20x stock solution. This was added to the slides for 10 minutes at RT and then washed in PBS. The slides were then ready for blocking and antibody staining.

2.1.6: Assessment of Cell Proliferation

With methods developed to detect 5-bromo-2-deoxyuridine (BrdU) using a monoclonal antibody (Gratzner 1982) it is now possible to detect BrdU, which is a pyrimidine analogue of thymidine. Replicating/proliferating cells incorporate BrdU, ensuring specific labelling of dividing cells only. Methods have been developed for the detection of BrdU in frozen, plastic and paraffin sections (Harms et al 1986, Farquharson and Loveridge 1990). It is necessary to preserve cell and tissue structure (Morstyn et al 1983) whilst still being able to expose the cell nuclei for antibody incorporation. Therefore, the most critical part of the BrdU immunolocalisation procedure is the hydrolysis step to partially denature cellular DNA in order that BrdU which has been taken up by the dividing cell is accessible to the monoclonal anti-BrdU antibodies (Moran et al 1985).

BrdU Labelling

At 20 days of age, 6 birds from each brooder in study 1 (section 2.1.1) (12 birds of each strain in total) were picked at random, intravenously injected with BrdU (Sigma) (25mg/kg), (4 injections) at four intervals over a 12 hour period, starting 24 hours before cull.

The birds were killed by cervical dislocation and sections of the right tibiotarsus were processed through to wax blocks as previously described (section 2.1.5.1). The wax embedded sections were dewaxed and rehydrated through a series of alcohols, placed into 1.5 M HCL for 30 mins at RT and washed 3x5 mins in PBS. Mouse-anti-BrdU (DAKO), primary antibody (100µl) diluted 1:50 in PBS, was placed directly onto the sections for 90 mins at RT in a humidity chamber. After further washing, 3 x 5 mins with phosphate-buffered saline (PBS), 100µl of secondary antibody, *FITC*-labelled goat anti mouse-IgG (Sigma), diluted 1: 50 in PBS, was added to the sections for 60 mins at RT in a humidity chamber. After a final wash, 3x5 mins in PBS, propidium iodide (Sigma) at 0.1mg/ml was used to counter-stain the sections (10 mins RT). Finally, the sections were mounted in Citifluor (Agar Scientific, Stansted, UK.). Samples were kept wrapped in tinfoil and stored at 4°C until analysed. Using confocal microscopy, (BIORAD MRC-500) four images were captured (BIORAD software version 7.0a for MRC-600) from each of two sections (sections 100 µm apart) from each diaphyseal sample. The first image was taken from the tension side of the bone, the second from the compression side of the bone, and the third and fourth from the two intermediate areas between these points (Fig 2.3).

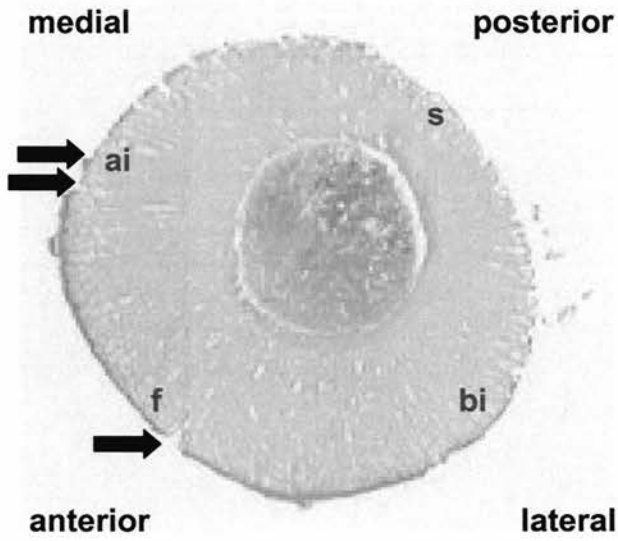


Figure 2.3 Orientated bone: the arrows indicate where cuts were made on the section immediately after dissection to determine bone orientation. The anterior is under tension and the posterior under compression. f=fast growing area of bone, s=slow growing area of bone, ai + bi= intermediate growth areas.

The total number of BrdU positive cells within each of the four sections round the periosteum were determined using ''Image Tool'' (version 2.01 Alpha 4, a public domain image analysis software package). The exact area of the image was taken to include the periosteum and the periosteal edge of the bone.

2.1.7: Blood vessel detection

Pilot studies used two different antibodies to detect laminin, (31 and 3H11), and one antibody to heparan sulphate (33) were used. All antibodies were obtained from Developmental Studies Hybridoma Bank (Iowa city, USA.).

Immunocytochemistry was used to localise laminin in basement membranes as a marker of blood vessels. Six chickens each of the fast and slow growing groups at 21 days were used (housing procedure 1, section 2.1.1). Sections were cut, dewaxed and rehydrated through a series of alcohols as previously described (section 2.1.5). Antigen sites were unmasked using pre-diluted Proteinase K (DAKO) for 8 minutes at RT, followed by

washing in PBS. Normal goat serum, diluted 1:50 in PBS, was placed directly onto the sections for 30 mins at RT followed by, without washing, primary laminin antibody (100µl) (3H11) diluted in PBS to 5µg IgG/ml. Sections were incubated in a humidity chamber overnight at 4°C. After further washing, 3x5 mins in PBS, 100µl of secondary antibody, *FITC*-labelled goat anti mouse IgG (Sigma), diluted 1:50 in PBS, was added to the sections for 60 mins at RT in a humidity chamber. After a final wash, 3x5 mins in PBS, they were finally mounted in Citifluor (Agar Scientific). Samples were kept wrapped in tinfoil and stored at 4°C until analysis. Using confocal microscopy, (BIORAD MRC-500) four images from two sections as previously described in BrdU labelling (section 2.1.6) (sections 100µm apart) were captured (BIORAD software version 7.0a for MRC-600) from the two strains of birds. These were all analysed using ''Image Tool''. The exact area of the image was taken to include the periosteum and the periosteal edge of the bone.

Controls

Control sections were treated exactly the same but using normal goat serum instead of the primary antibody for laminin.

2.1.8: Determination of porosity

Serial sections, 7 µm in thickness, were cut from decalcified wax embedded blocks, processed through alcohols and stained for 30 seconds with 0.1% w/v Toluidine blue O in PBS buffer (pH 5.5). The sections were dehydrated through alcohols and mounted with DePeX (VWR International, Poole, UK). These were all analysed using ''Image Tool''.

2.1.9: Determination of osteon infilling

Serial sections, 7 μm in thickness, were cut from decalcified wax embedded blocks, processed through alcohols and stained for 30 seconds with 0.1% w/v Toluidine blue O in PBS buffer (pH 5.5). The sections were dehydrated through alcohols and mounted with DePeX. These were all analysed using ''Image Tool''.

2.1.10: Mineral apposition rate

Knowledge concerning the rate of bone formation has been gained by the use of *in vivo* markers such as tetracyclines (Tapp 1966, Frost 1969, Hansson et al 1974). Calcein works on the same principle as the antibiotic tetracycline. Both are known to form complex with calcium ions at the surfaces of newly formed apatite crystals. This marks the site of initial mineralisation (Sandhu and Jande 1981). The uptake of two fluorescent labels over a known time interval allows the determination of the mineral apposition rate (MAR). Fluorochrome labelling has been used to diagnose and study bone disorders in both humans and animals (Frost 1969). This procedure has been used extensively to compare bone growth rates in chickens fed various dietary regimes (Newbury et al 1988, Hudson et al 1993) but it has been utilised less often to compare bone growth rates between different strains of chickens (Wise 1970, Poulos et al 1978, Vitirovic et al 1995).

Calcein labelling

Ten birds (kept under housing method 2, section 2.1.2) from both the fast and the slow strain were administered two intraveneous injections of calcein fluorochrome (Sigma) at 10mg/kg/bw in 0.5ml sterile water containing 20mg/kg/bw sodium bicarbonate. The injections were 72 hrs apart (18 and 21 days of age) and the birds

were culled 8 hrs after the last injection. Tibiotarsi were dissected out of the bird (tissue extraction section 2.1.3) and diaphyseal sections prepared as previously described. Frozen sections (10μm in thickness) were cut, and examined by confocal microscopy (BIORAD MRC-500). Four images from each of the two sections as previously described in BrdU, section 2.1.6) (sections 100 μm apart) from each bone were analysed using ''Image Tool'' The exact area of the image taken just inside the periosteal edge of the bone.

2.1.11: Cement line staining

Serial sections, 7 μm in thickness, were cut from decalcified wax embedded blocks, processed through alcohols and stained for 30 seconds with 0.1% w/v Toluidine blue O in PBS buffer (pH 5.5). The sections were dehydrated through alcohols and mounted with DePeX.

2.1.12: Collagen fibril orientation

Frozen tissue which had been dissected according to section 2.1.3, were cut (section 2.1.5.2) to observe collagen fibre orientation. This was observed under cross polarised light introduced above and below the bone section. This was carried out on a Leica DMRB fluorescent microscope with the pictures captured to computer using a 3cld sony camera.

2.1.13: Tartrate Resistant Acid Phosphatase (TRAP) Staining

Frozen sections were cut according to chapter 2 (section 2.1.5.2) and mounted on poly-l-lysine slides. Slides were incubated for 2 minutes at 37°C in 100 mls of citrate buffer pH 4.5 which contained 50 mg Napthol-AS BI-Phosphate (Sigma) and 230 mg

of tartrate (Sigma). Slides were then rinsed in cold 50 mM sodium fluoride for 5 minutes. They were further incubated for 15 minutes in 0.1 M acetate buffer containing 1 mg/ml fast garnet (Sigma). After washing in dH_2O the slides were mounted in aquamount (Agar Scientific).

2.1.14: Bone strength and stiffness analysis

The tibiotarsi were cleaned of all meat including the periosteal membrane, taking care not to damage them in any way. Bone length and width were measured using callipers, and the values entered into the computer with strain and chicken number before each bone was tested. Mechanical properties were then measured using an LRX materials tester (Lloyds Instruments, UK) with a cell load value of 2500 N, and a cell calibration of 105.5%. The materials tester was run under remote control of a computer running the software package ''Nexygen 2.2'' via the software package ''LrLrxConsole'' (both Lloyds Instruments, UK), and it was this system into which the bone length and width measurements were put. The compress to rupture test within the Nexygen programme was used in combination with a three-point-bend jig on the materials tester. Three-point-bending consists of two curved lower rests, each of 10 mm in diameter and spaced 30mm apart, on which the tibiotarsi was balanced. A third crosshead pin of the same dimensions applied a downward force central to the bottom two pins. The tibiotarsi were always placed with the distal end to the left with the flat distal surface pointing upwards to ensure consistency in testing. The test was run, to the point where the bone broke/ruptured and the Nexygen programme calculated the maximum load applied to the sample before it ruptured. Load-deformation curves were generated and several biomechanical characteristics were determined from these curves within the programme: i) yield load (a measure of the

elastic limit of the bone); ii) resilience (the energy absorbed by the bone until the yield point is reached;) iii) peak load (a measure of the maximum force that the bone withstood before fracture); iv) toughness (a measure of the work energy that is required to fracture the bone). These characteristics are generated by the computer programme.

2.1.15: Live/dead cell detection

(after: Roach H, Southampton University, UK. Unpublished, kind donation of their laboratory techniques.)

Bone sections (section 2.1.4) were placed into a dark container containing culture medium (DMEM) and two probes: celltracker green (CMFDA) and ethidium homodiner-1(EthD-1), (Molecular Probes). CMFDA (50 μg) was dissolved in 50 μl of ethanol and added to 25 μl of EthD-1 (supplied as 1 mg and dissolved in 1 ml dH_2O) and 4 ml of DMEM (Gibco, Paisley, UK). The probes were kept in the dark and placed in an incubator at 37°C. Two unfixed samples from each bone were taken, one of which was incubated in the probes for 5 hours whereas the other was left in the probe solution for 24 hours. Both were kept at 37°C throughout the incubation period and thereafter the tissue was washed with PBS, fixed in 70% alcohol and decalcified before processing into paraffin blocks as previously described (section 2.1.5.1). Samples were kept in the dark at all times.

2.2: Cell culture *In Vitro* studies

2.2.1: Osteoblast extraction from tibiotarsi

(After Nijweide et al 2003)

At 21 days of age, chickens were culled by cervical dislocation, and submerged in virkon (VWR International Ltd, Lutterworth, UK) for 10 minutes to disinfect the whole bird. All equipment used was autoclaved prior to use in a bench top clinical autoclave: Series 2100 (Prestige Medical International, Derotstown, Ireland) and kept in 70% alcohol during use. Tibiotarsi were removed using a change of scalpel blade for each bone and separate blades were used to cut skin and muscle. Dissected bones were stored in separate sterile containers and further processing carried out in a class III flow hood. Both ends containing the epiphysis and metaphysis were cut off and discarded using bone cutters and any adhering muscle and periosteum were removed from the diaphyseal shaft using sterile swabs. Using a syringe and needle the bone marrow was flushed out using sterile PBS with 1% fungizone. The bones were then chopped into small pieces using bone cutters. The bone fragments from each bird were then incubated at 37°C on a shaker for 20 minutes in 4 ml of digestion solution, (appendix 1). The supernatant was transferred to a universal containing 700 µl of fetal bovine serum (FBS) (Gibco). The bone fragments were washed with 3 ml DMEM (without FBS), and the wash solution was added to the supernatant. This was population 1. The cell suspension was centrifuged at 2500 g for 5 minutes and the supernatant was discarded and the cell pellet re-suspended in 1ml of complete culture medium (cCM) (appendix 1). This extraction procedure was repeated until 4 populations were obtained. Populations were combined to form 2 cultures of cells, population 1+2 and population 3+4, each set up in T75 culture flask (Gibco) with 20 mls cCM. The flasks were left untouched for 3-5 days to allow the cells time to attach before the medium was changed.

2.2.2: Passage of Cells

When the cells were approximately 70-80% confluent, the cells were treated with collagenase first, because they were grown in the presence of ascorbic acid. The cells were first rinsed in 2x10 ml with DMEM, and then incubated at 37°C for 2 hours with 25 U/ml of collagenase and 2 mM of $CaCl_2$ in 10 ml of DMEM with agitation every 30 minutes. Digestion was terminated by removing the medium, and the cells were washed 2x in $Ca^{2+}Mg^{2+}$ free PBS (Sigma) (10 ml). Trypsin/EDTA (Sigma) solution was added for 5 minutes to remove the cells (5 ml for a T75 flask). The cell suspension was added to a universal and the flask washed out with 5 ml of cCM, which was added to the universal. The cells were centrifuged at 2000 rpm for 5 minutes and the medium discarded. The cell pellet was resuspended in 1 ml of cCM and the volume was made up to 5 ml before a cell count obtained using a haemocytometer. Cells from each bone were plated down into a number of T175 flasks, with the cells from the slow growing strain plated at a lower density (S=10 000 /cm^2, F=15 000 /cm^2) as these visibly grew at a faster rate than those from the fast growing strain.

2.2.3: Plate set up for assays

After a second passage, sufficient cells were available for experimentation. Osteoblast cells from both fast and slow growing strains were plated at a density of 20 000 /cm^2. One of the cultures from the slow growing bones was lost to bacterial contamination and therefore the experiment was carried out with 3 slow and 4 fast cell phenotypes. For thymidine and the determination of hydroxyproline analysis 48 well plates with five wells per sample were used. For the ALP activity, 24 well plates were used, with four wells per sample. For all assays, plates were set up to study 3 maturational time points in osteoblast differentation; pre-confluent, confluent, and

post-confluent. With pre-confluency taken at 3 days post plating, confluency taken at 6 days post plating and post-confluency taken at 11 days post plating.

2.2.4: Bone Collagen (hydroxyproline) Assay

Sample Hydrolysation

Osteoblasts cultured in 48 well plates were scraped in 300 µl dH_2O using a 1 ml pipette tip and transferred to capped Eppendorfs with equal amounts of neat (12 M) HCL. Eppendorfs were placed in an oven at 107°C for 24hrs to hydrolyse the collagen into individual amino acids. The hydrolysate was then transferred to glass vials and the Eppendorfs were washed out with 400 µl water which was than combined with the hydrolysate. This was vacuumed down at 50°C, reconstituted in 300 µl of water and spun down at 2000 rpm for 5 minutes to precipitate any carbon material. Samples were stored at 4°C until analysed.

Standards

A range of standards was produced from a stock hydroxyproline solution of 500nmoles/ml. The stock was diluted 1:10 with dH_2O to give a top standard of 50 nmoles/ml, or 6.55 µg/ml (1mole=131g). Serial dilutions were performed to produce a range of standards with concentrations of 6.55, 3.3, 1.6, 0.8, 0.4 and 0.2 µg/ml.

Hydroxyproline (OHP) assay

(After Creemers et al 1997)

To each well of a 96 well microtitre plate, 60 µl of sample/standard was added in duplicate. To this 20 µl of assay buffer (appendix 1) and of 40µl chloramine T

(appendix 1) was added and incubated at RT for 15 minutes. After the addition of 80 µl DMBA reagent (appendix 1) to each well the plate was covered with parafilm to prevent evaporation and placed in a shaking water bath at 60°C for 20 minutes. The cover was removed immediately and the plate allowed to cool on ice for 5 minutes and absorbance was read at 560nm using a Dynatech MR7000 plate reader in conjunction with Revelations version 3.2 software package.

2.2.5: Proliferation assay – ^{3}[H]-thymidine uptake

Thymidine stock (Amersham) was 10 uCi/ml, therefore 20 µl of stock was added to 1 ml of medium to give a final concentration of 0.2 uCi/ml. The addition of thymidine was carried out 18 hours before the end of each time point. At each time point the medium was removed and the cell layer was washed twice for 5 minutes with DMEM to remove any unbound thymidine. The cells were fixed with 1 ml of 5% cold trichloroacetic acid for 15 minutes and then washed twice with PBS. 0.1M NaOH was added and left for 10 minutes to lyse the cells. All of the NaOH was added to a scintillation vial and the wells washed out with 200µl of PBS which was also added to the vial. Scintillant (Fisons chemicals, Loughborough, UK) (3ml) was added and mixed well before reading on a liquid scintillation counter (Wallac 1410 model, PerkinElmer, Beltsville, USA.).

2.2.6: Alkaline phosphatase (ALP) activity

Osteoblasts cultured in 24 well plates had the medium removed and were washed twice with PBS. The cells were scraped in 300µl of triton-x-100 (0.1%) using a 1 ml pipette tip and transferred to an Eppendorf before centrifugation for 5 minutes at 12 000 g at 4°C. Sample or standards were added (50 µl) in duplicate to a 96 well plate.

Pre-heated alkaline phosphatase AMP buffer (100 µl) (Thermo Trace Ltd, Melbourne, Australia) (37°C) containing p-nitro-phenyl as a substrate was added. This was read immediately at 405 nm on a pre-heated plate reader (Dynatech MR7000), in conjunction with Revelations version 3.2 software package. Absorbance was recorded every minute for 30 minutes.

Standards

A range of standards was produced from a stock p-nitro-phenol (Sigma) solution. The stock was diluted 1:10 with distilled water to give a top standard of to 500 µM. Serial dilutions were performed to produce a range of standards with concentrations of 500, 250, 125, 62.50, 31.25, 15.63, 7.81, 3.90 and 1.95 µM.

2.2.7: Immunoblotting

Protein Extraction

(after: Wojcik et al 1999)

The medium was removed from the cultures and the cells were scraped with ice cold PBS before centrifugation at 2500 g for 5 minutes. The pellet was then resuspended in 1.5 ml of RIPA buffer (appendix 1) containing protease inhibitors and shaken on a table shaker for 30 minutes at 4°C. After centrifugation at 12000 g for 1 minute the supernatant was stored at -80°C until analysis.

Cell Protein assay

Protein lysate samples were diluted 1:60 with dH_2O and 160 µl of sample or standard was added in duplicate to a 96 well microplate. To this 40 µl of detergent compatable Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Hemel Hempstead, UK) was

added. This was mixed thoroughly and left for 5 minutes before the optical density of 595 nm was determined on a plate reader (Dynatech MR7000) in conjunction with Revelations version 3.2 software package.

Standards

A range of protein standards was produced from a stock solution of gamma-globulin at 2 mg/ml. The stock was diluted with dH_2O to give a top standard of to 90 μg/ml. A range of standards were produced with concentrations of 90, 80, 70, 60, 50, 40, 30, 20, and 10 μg/ml.

Western Blotting

(after: Invitrogen methodology)

Sample preparation and reduction

Individual protein samples from fast and slow chickens cell cultures were added to 10 μl of loading buffer and 3 μl reducing agent. Both loading buffer and reducing agent were supplied by Invitrogen (Paisley, UK) and diluted for use. Samples were diluted with dH_2O to contain 30 μg of protein. All samples were denatured by heating for 10 minutes at 70°C follwed by vortexing and loading onto the gel.

The gel

Samples were loaded immediately onto NuPage 10% Bis-Tris polyacrylimide precast gels (Invitrogen) which were submerged in MOPS running buffer in a tank (Invitrogen). The gels were run at 200 V for 50 minutes or for 1 h 15 minutes if two tanks were running simultaneously.

Gel transfer

The gels were set up for transfer according to the manufactures guide with blotting pads, filter paper and Hybond-P nitrocellulose transfer membrane (Amersham Biosciences, Little Chalfont,m UK) and placed in the tank with transfer buffer (Invitrogen). This was run at 25 V for 1 hour.

Antibody binding

Five different primary antibodies were used; one to collagen type I (kindly donated by Victor Duance, Cardiff University, UK; - goat anti bovine type I); two to human bone sialoprotein (LF-83 & LF-119) known to react with chick protein, and two to chicken osteonectin (LF-8 & LF-45) (kindly donated by Fisher L W, NIDCR, Bethesda, USA). The transfer membrane was placed in blocking buffer made up with 5% marvel milk powder in TBS-tween 20 (appendix 1). It was either blocked for 1 hour at 37°C in a shaking incubator or left overnight at 4°C. After washing with TBS-tween 20 for 3x 5 minutes the primary antibodies were diluted in blocking buffer to a 1:2000 dilution, and incubated for 1 hour at 37°C in a shaking incubator. After further washing in TBS-tween 20 for 3x 5 minutes appropriate secondary antibodies were added and incubated for 1 hour at 37°C in a shaking incubator. Goat anti- rabbit-IgG-peroxidase (DAKO) diluted 1:2000 with PBS was used in conjunction with the BSP and osteonectin antibodies and goat anti-sheep-IgG-peroxidase antibody (Sigma) diluted 1:500 with PBS was used in conjunction with the collagen type I antibody. A final wash of TBS-tween 20 for 1x 20 minutes and 1x 1 hour was carried out before signal detection.

Band detection

Detection was carried out using an ECL plus Western Blotting detection system (Amersham Biosciences) and exposure to Hyperfilm ECL (Amersham Biosciences) in a dark room was for 5 seconds before running through a film processor.

2.2.8: RNA extraction – cell culture

Medium from cell cultures was decanted and the cells immediately scraped in 1ml of Ultraspec (ams Biotechnology Ltd, Adingdon, UK), transferred to Universals and stored at -80°C. On thawing, the cells were homogenised and syringed through a 25 G needle to ensure total break up of the cells. The homogenate was transfered to a 1 ml Eppendorf tube and left on ice for 5 minutes. To this, 200 µl of chloroform was added and shaken vigorously for 15 seconds, vortexed and left on ice for a further 5 minutes. This was followed by centrifugation at 12000g for 15 minutes at 4°C. The upper aqueous phase was carefully removed to a sterile Eppendorf to which 250µl of isopropanol and 50 µl RNA tack resin was added. The mixture was vortexed and centrifuged for 1 minute at 12000 g. The supernatant was discarded and the pellet washed twice with 1 ml of 75% ethanol, final traces of ethanol were removed with a small tip, and the pellet allowed to dry at RT for 30 minutes. The RNA was eluted by resuspending the pellet in 100 µl of nuclease free water vortexed, and centrifuged at 12000 g for 1 minute. The supernatant was transferred to a new Eppendorf and allowed to sit on ice for 1 minute and centrifuged again at the same speed. To each volume of 100µl of RNA, 100µl of 10x DNAse 1 buffer, 3 µl of RNAse Inhibitors, 5 µl DNAse I were added and mixed gently. This was heated to 37°C for 1 hour, after which, 12 µl of inactivation reagent was added and the mixture gently flicked before

centrifugation at 12000 g for 1 minute. The RNA now in the supernatant, was transferred into a sterile Eppendorf and stored at -80°C.

2.2.9: Reverse Transcription- Polymerase Chain Reaction

Reverse transcription- polymerase chain reaction (RT-PCR), was carried out using a one-step RT-PCR kit (Qiagen, Crawley, UK). The concentration of RNA was determined on a spectophotometer at 260/280 nm. RNA at a concentration of 1 ng in a 10 µl reaction, which contained a final volume of 400 µM of dNTP mix, 0.6 µM of each primer, one step buffer and RT-PCR enzyme mix. Target genes, and optimum annealing temperatures and cycle numbers are listed in (Table 2.1).

After the addition of loading buffer all RT-PCR samples were loaded onto a 1.5% agarose gel containing ethidium bromide (at 2.5 µl/100 ml/gel), and run at 120 V for 50 minutes. As a control a blank was run using sterile water instead of RNA. Gels were examined on an ultraviolet lightbox and pictures taken with Multi-analyst version 1.1 software package (Bio-Rad). Densitometry was carried out using Quantity One version 4.2.2 software package (Bio-Rad).

2.2.10: Laser Capture Microscopy (LCM)

Tissue preparation

Bone sections collected in tissue extraction (2) (section2.1.4), were sectioned and de-waxed under RNAse free conditions. Staining was carried out for 30 seconds using Arcturus LCM stain (Arcturus, Braintree, UK) and then dehydrated back to xylene. The slides were air dried in a class III hood and immediately placed into a dessicator until analysis. (Two sections were cut from each sample).

Table 2.1 Target genes for RT-PCR from cell cultures and LCM

Gene No.	Target gene	Forward primer	Reverse primer	Annealing temp °C	No of cycles	Product size	source
1	GAPDH	CAC GCC ATC ACT ATC TTC	CAC AAT GCC AAA GTT GTC	45	35	300	Westbroek et al (2001)
2	Seratonin receptor (5-HT2BR)	GAT CAA CAA GCC ACC TCA AC	ATA CCT GCT GAA AGC CTC C	58	35	420	Westbroek et al (2001)
3	Collagen type I (Col 1A1)	AAC CCG GCT GAT GTC GCC ATC CAA C	GGG CCG ATG TCA ATG CCA AAT TC	60	25	351	Nakata et al (1992)
4	Osteonectin (ONN)	AAG ATG TAG AGG AGA TCG TCG CAG	CAT TCC TCC AGG GCG ATG TAC	60	18	439	Nurminskaya et al (2003)
5	Bone sialoprotein (BSP)	CCG GTA CTA CCT GTA CCG CTA CG	TCC CAC TGT CAC CTC GTA CTC	62	35	480	Nurminskaya et al (2003)
6	Osteopontin (OPN)	TGC CAG GAA GCT CAT TGA GGA TG	GCG TCT ACA TTT ACA AAC ACA CGT C	60	25	419	Kuykindoll et al (1999)
7	Osteocalcein (OCN)	ATG CTC GCA GTG CTA AAG CCT	TTA TTT CTG TCC ATC CTT CGC G	52	35	318	Nurminskaya et al (2003)
8	Runx2	ACT TTG ACA ATA ACT GTC CT	GAC ACC TAC TCT CAT ACT GG	58	35	364	Drissi et al (2003)
9	ERα	AGC AAC AAA GGA ATG GAG CA	TAC GCT GCT GGG TTT CTC AT	58	35	320	Cooke V E (2004)
10	Collagen type II	GCA GAG ACC ATC AAC GGC GGT	CAG GCG CGA GGT CTT CTG CGA	58	55	323	Farquharson et al (2001)
11	Collagen type X	AAG GGG CCA CCA CAC TTT CTA	TTC TCC AGG CTT CCC TAT CCC	58	55	399	Farquharson et al (2001)
12	Glutamate receptor (NMDA)	CTG GTG ACA ACA GGG GAA CT	ACA ATA CCT CCA GCC ACC AG	Range 50-62	Range 25-75	239	Personally designed
13	IGF1-receptor	CTC TTC CCC AAC CTC ACG GTC A	GCT TCT CCT CCA TCG TTC CTG G	Range 50-62	Range 25-75	274	Armstrong and Hogg (1992)
14	eNOS	CCT TCA GTG GCT GGT ACA	ATG GGG GGC ACG ATC CA	Range 50-62	Range 25-75	301	Fujii et al (1998)
15	IGF-1	TTC TTC TAC CTG GCC TGT G	CAT ACC CTG TAG GCT TAC TG	Range 50-62	Range 25-75	147	Baudet et al (2003)

LCM

Slides were removed from the dessicsator one at a time and gently pressed against tissue prepartion strip (Arcturus) used to ensure removal of any dust/ loose particles. Using Capsure HS LCM caps (Arcturus), two areas from one section were targeted by the laser to remove osteoblasts from within newly formed primary osteons situated within the periosteal surface. One cap per slide was used with the cap being placed immediately into a RNAse free Eppendorf. Using RNeasy micro extraction kit (Qiagen), 40 µl of RLT buffer (supplied in kit) was placed into the cap within the Eppendorf tube at RT for 30 minutes. The caps within the Eppendorfs were then centrifuged at 12000 g for 1 minute to pellet the contents of the cap into the Eppendorf. The volume was made up to 75 µl with RLT buffer. After the addition of 70% ethanol (75 µl), the contents were then loaded into Rneasy MinElute Spin column (supplied in kit) and centrifuged at 8000 g for 30 seconds with the flow-through being discarded. Buffer RW1 (supplied in kit) was added (350 µl) to the spin column which was centrifuged at 8000 g for 30 seconds, and the flow-through disgarded. Dnase I incubation mix was then added (80 µl) and incubated at RT for 15 minutes and this was then washed through with buffer RW1 (350 µl) at 8000 g for 30 seconds. The flow-through was again discarded. 500 µl of buffer RPE (supplied in kit) was added to the spin column and centrifuged at 8000 g for 30 seconds. The flow-through was discarded. 500 µl of ethanol (80%) was added to the spin column and centrifuged at 8000 g for 2 minutes and the flow-through discarded. The columns were further centrifuged at 12000 g for 5 minutes and 14 µl of RNAse-free water was added to elute the RNA after centrifugation at 12000 for 1 minute. The RNA was stored at –80°C until analysis.

RT-PCR

RT-PCR was carried out using 50 ng/reaction according to the same method described in section 2.2.9.

Validation of LCM Protocol

Chicken growth plates decalcified and processed using an RNAse free protocol were targeted by the laser to extract cells from the proliferating (cap1) and hypertrophic zone (cap 2). RNA from both cell types were assessed for collagen II and collagen X expression by RT-PCR reaction using 50 ng/reaction of RNA. Primers for collagen II and collagen X were used to amplify RNA according to Table 2.1.

2.2.11: Statistical analysis

Statistical analysis was carried out by ANOVA using Genstat statistical package for windows (Lawes Agricultural Trust, Rothamsted Experimental Station). All data are represented as mean ± SEM. Where error bars are not present on graphs the error size was too small to be shown.

Chapter 3: Determination of the cause of increased cortical porosity in the fast growing broiler. A histomorphometric study.

3.1: Introduction

To date, research into pathologies of the avian skeleton has been predominantly concerned with growth plate cartilage and disorders of longitudinal bone growth such as rickets and tibial dyschondroplasia (Farquharson and Jefferies 2000). In contrast, relatively little data exists on the underlying mechanisms that result in a high incidence of cortical bone fractures (Kestin et al 1992).

As first proposed in Wolff's law (Wolff 1892) bone architecture of both the mature and growing skeleton adapts to withstand the extremes of functional load-bearing (Lanyon 1980, Carter et al 1991, van der Meulen 1993). In response to increased loads during growth, bones enlarge their circumference, increasing their cross sectional moment of inertia (CSMI) and thereby their bending strength, by the rapid laying down on the periosteal surface of *de novo* primary bone (Martin et al 1998). This primary bone can take the form of circumferential lamellae that are orientated parallel to the bone surface with incorporated primary osteons or plexiform bone which has a "brick wall" appearance and is made up of a mixture of woven and laminar bone (Martin and Burr 1989). Plexiform bone is associated with rapid bone formation in large fast growing animals and has not been noted in cortical diaphyseal bone of the broiler chick, which is characterised by osteonal bone formation (Thorp and Waddington 1997, Williams et al 2000 b) The development of primary osteons within circumferential lamellar bone occurs by altered osteoblastic activity and differential bone growth resulting in longitudinal depressions on the periosteal surface. The eventual entrapment of blood vessels and periosteal osteoblasts and the subsequent infilling of the canals by concentric laminae of bone results in the formation of the primary osteon (Banks 1986).

It has been previously reported that, throughout the productive life span, cortical bone from a heavy modern strain of bird was consistently more porous and less well mineralised than that from a slower growing control strain (Williams et al 2000 a, b). It has previously been suggested that a disruption to the Ca:P ratio of the bone mineral might lead to weakness in the bone crystal lattice therefore contributing to leg problems (Thorp and Waddington 1997). Additional studies by this group have also indicated that bone breaking strength and bone stiffness is lower in the fast growing modern strain bird (Williams et al 2004).

It is unclear, however, if the cortical bone morphology observed in the modern fast growing broiler is related to the rapid radial expansion of load-bearing bones necessary to withstand the rapid increases in body weight. In addition, it is also uncertain whether the porosity observed is due to increased bone resorption within an osteon remodelling unit or a reduced bone production rate in the infilling process during primary osteon formation.

3.2: Aims

The present study aimed to confirm and extend the differences previously observed in bone porosity between modern meat-type and control strains of chicken (Williams et al 2000 a) and to make preliminary investigations on the mechanical properties of bone. In addition, some of the possible cellular mechanisms for the increased porosity observed in the modern strain of chickens were examined. A genetically distinct fast growing strain, and a control, slow growing strain were studied.

3.3: Methods

3.3.1: Birds, Housing and Tissue

Both fast and slow growing strains of chickens were fed and housed according to chapter 2 (section 2.1.1). The birds were all culled at 14 and 42 days of age. The right tibiotarsus was dissected out according chapter 2 (section 2.1.3). The left tibiotarsus was cleaned of all muscle tissue and periosteum and used for bone mechanical analysis. The histologically processed bone sections from the right tibiotarsi of 14 and 42 day-old chickens were used to study cortical bone porosity, osteocyte density, primary osteon infilling and collagen orientation (polarised light). The bone sections utilised for cement line staining were from 14 and 42 day old tibiotarsi that had been decalcified and embedded in paraffin wax. This latter tissue was available from a previous study (Williams et al 2004).

3.3.2: Staining of Cement Lines

Various staining methods were tried according to papers discussed in section 3.5, although none were specifically prescribed for decalcified wax embedded sections. Staining was carried out according to chapter 2 (section 2.1.9). The toludine blue O protocol was the most effective for the staining of cement lines (section 3.4.3), and was also a suitable stain for the identification of primary osteons and osteocytes within the cortical bone.

3.3.3: Calcein labelling

Optimisation of injection times

Four fast growing chickens, each of seventeen, eighteen, nineteen and twenty days of age were injected with 10 mg/kg of calcein, then again at twenty-one days and culled by cervical dislocation eight hours after the second injection. This gave a time span

between injections of ninety-six, seventy-two, forty-eight and twenty-four hours respectively.

Birds and housing

Ten birds of the fast and the slow strain were kept in floor pens. They were administered two intraveneous injections of calcein fluorochrome 72 hrs apart (18 and 21 days of age) and the birds were culled 8 hrs after the last injection.

3.4: Results

3.4.1: Body weight and bone dimensions

At 42 days of age there was a significant difference in body weight between fast and slow growing chickens. The fast growing chickens were double the weight of the slow growing chickens where F = fast and S = slow (F=2440 ± 1.8 g, S=1224 ± 2.1 g; P > 0.001), (Fig 3.1).

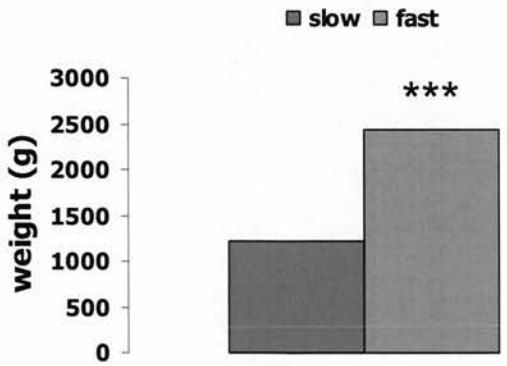


Figure 3.1. Body weights of slow (n=10) and fast (n=10) growth chickens at 42 days of age. Error bars were too small to be visible on the graph. Data are shown as mean ± SEM, ***P<0.001.

Bone diameter (F = 7.16 ± 0.8 mm, S = 5.36 ± 0.3 mm; P < 0.001) and cortical width (F = 0.702 ± 0.1 mm, S = 0.479 ± 0.08 mm; P < 0.01) were increased in the rapidly growing birds.

3.4.2: Mechanical properties

Bone stiffness of the tibiotarsi was higher in the fast growing chickens than that of the slow growing chickens both at 14 days (F = 77 ± 4.3 N/mm, S = 44 ± 2.1 N/mm; P > 0.001) and 42 days (F = 363 ± 4.8 N/mm, S = 285 ± 4.6 N/mm; P < 0.001) (Fig 3.2). Once adjusted for body weight, bone stiffness was lower in the fast growing chickens than that of the slow growing chickens at 14 days (F = 60 ± 0.7 N/mm, S = 99 ± 0.6 N/mm; P < 0.01). By the age of 42 days there was no significant difference seen between the strains (F = 363 ± 1.8 N/mm, S = 361 ± 1.1 N/mm) (Fig 3.3).

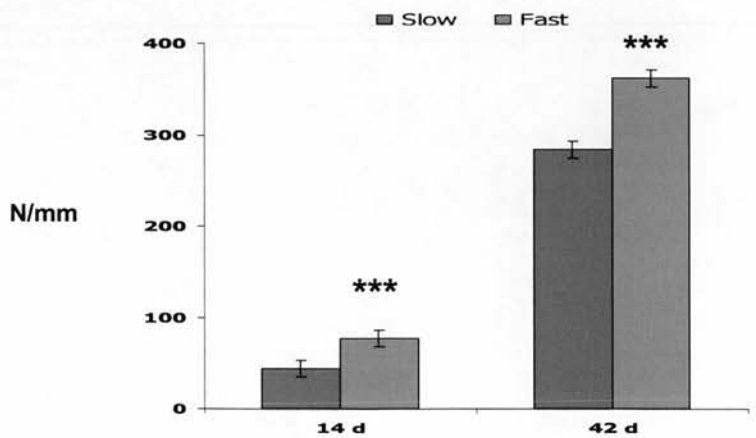


Figure 3.2 Tibiotarsi stiffness of fast (n=10) and slow (n=10) growth chickens at 14 and 42 days before adjustment for body weight. Data are shown as mean ± SEM, ***P<0.001.

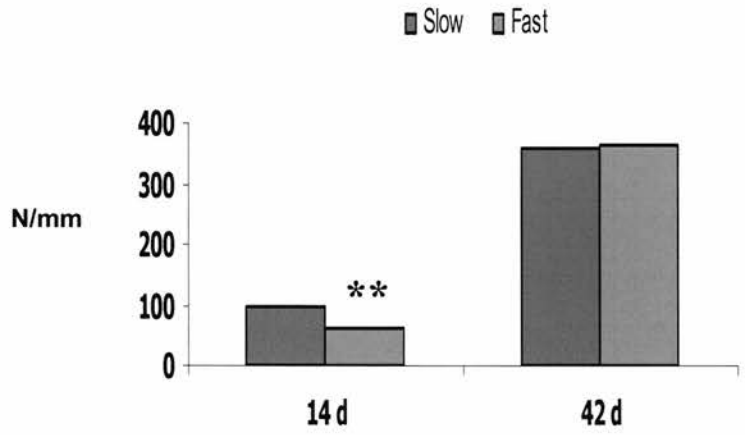


Figure 3.3 Tibiotarsi stiffness of fast and slow growth chickens at 14 and 42 days after adjustment for body weight. Error bars were too small to be visible on the graph. Data are shown as mean ± SEM, ** P<0.01.

Breaking strength of the tibiotarsi was also higher in the fast growing chickens compared to that of the slow growing chickens at 14 days (F = 55 ± 1.7 N, S = 104 ± 1.3 N; $P < 0.001$) with no significant difference at 42 days (F = 454 ± 1.6 N, S = 448 ± 0.8 N) (Fig 3.4). After adjustment for body weight the breaking strength was lower in the fast growing chickens at 14 days (F = 63 ± 1.4 N, S = 83 ± 1.2 N; $P < 0.01$) as was the case at the 42 days of age (F = 296 ± 2.3 N, S = 362 ± 0.5 N; $P < 0.01$) (Fig 3.5).

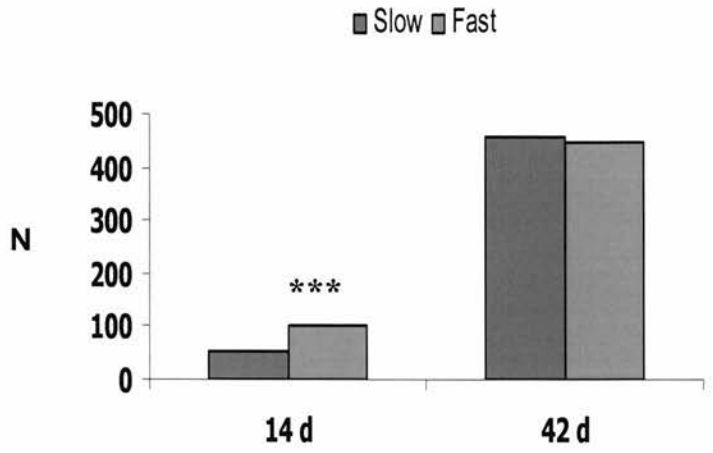


Figure 3.4 Tibiotarsi breaking strength of fast (n=10) and slow (n=10) chickens, at 14 and 42 days of age, before adjustment for body weight. Error bars were too small to be visible on the graph. Data are shown as mean ± SEM, ***P<0.001.

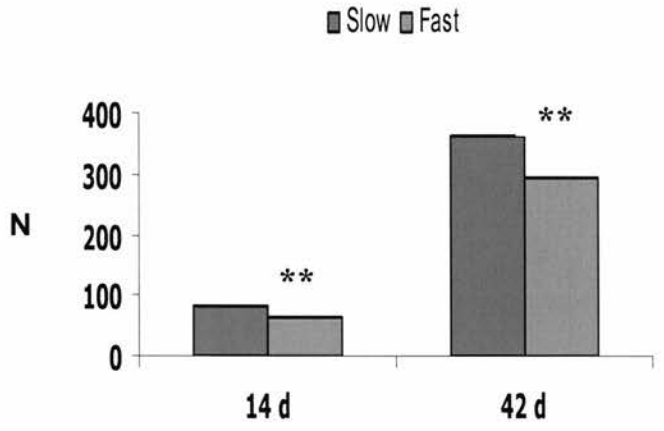


Figure 3.5 Tibiotarsi breaking strength at 14 and 42 days of age after adjustment for body weight. Error bars were too small to be visible on the graph. Data are shown as mean ± SEM, ** P<0.01.

3.4.3: Determination of cortical bone porosity

Cortical porosity was elevated periosteally in the fast growing chickens compared to that of the slow growing birds at both 14 days (F = 55.23 ± 1.1%, S = 47.89 ±

0.02%; P < 0.05) and 42 days of age (F = 35.64 ± 0.01%, S = 26.86 ± 0.02%; P < 0.01) (Fig 3.6). There was no significant difference in porosity endosteally between the two strains (Fig 3.7).

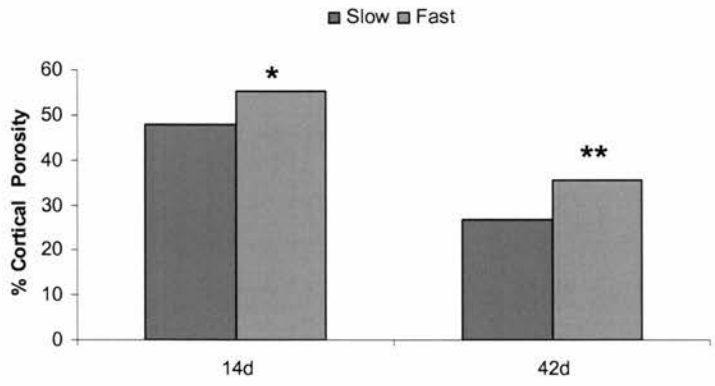


Figure 3.6 Cortical bone porosity at the periosteal edge of fast (n=10) and slow(n=10) growth chickens at 14 and 42 days of age. Error bars were too small to be visible on the graph. Data are shown as mean ± SEM, * P<0.05; ** P<0.01.

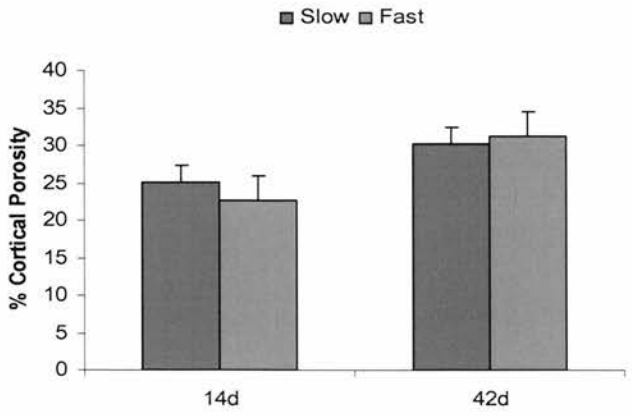


Figure 3.7 Cortical bone porosity at the endosteal edge of fast and slow growth chickens at 14 and 42 days of age. Data are shown as mean ± SEM.

3.4.4: Determination of secondary remodelling

At 14 days of age, no cement line staining was seen within the fast growing strain around or within any of the primary osteons. Large cigar shaped pores were seen coming in from the periosteal surface at the anterior side. This lack of cement line staining implies no secondary remodelling was present in the fast growing strain. Within the slow growing strain, at 14 days of age, small areas at the edge of the endosteal surface were seen to have cement lines present but these did not cover the whole endosteal surface. The presence of cement lines was dependent on whether the bone was under tension or compression. Figure 3.8 shows the posterior endosteal surface which is under compression. There was no evidence of secondary remodelling at the periosteal surface due to the absence of cement lines.

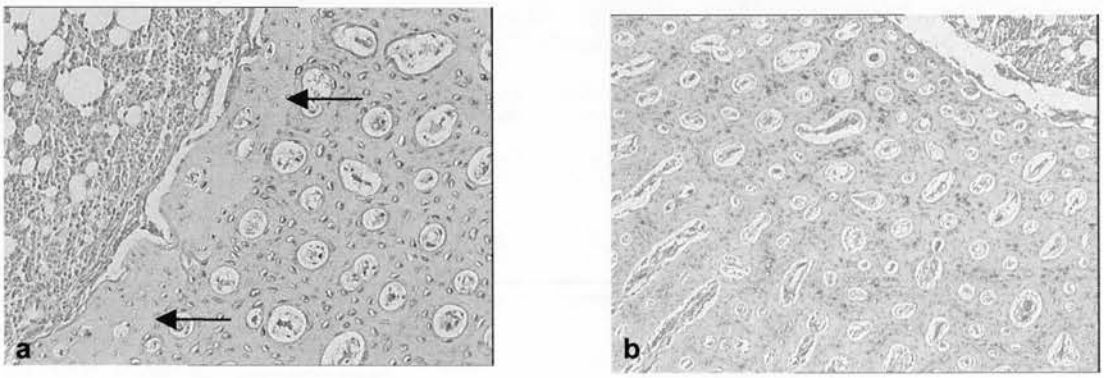


Figure 3.8 Secondary remodelling in 14 day old chickens within the slow growing strain (a) with the arrows indicating the tide line and secondary remodelling sites at the endosteal surface. In the fast growing strain (b) no tide line or secondary remodelling sites were seen.

At 42 days of age, cortical bone of the fast growing strain was seen to contain both cement lines and a tide line. Cigar shaped pores coming in from the periosteal surface were visible on the anterior side, but not as large in shape as those found in

the 14 day-old fast strain. Again these cigar shape pores appeared to become smaller before reaching the endosteal surface for re-absorption at the endosteal edge.

Within the fast growing strain the tide line was positioned a quarter of a way down from the periosteal surface on the posterior side, running round the whole of the cortex in an oval fashion and getting closer to the endosteal surface at the anterior side which was under tension (the fastest growing, most porous area of the bone) Cement lines were only seen below the tide line, towards the endosteal surface.

The cortical bone of 42-day-old slow growing chickens also had both cement lines and a tide line. This was present right out at the periosteal edge on the posterior side (under compression, slowest growing area of the cortex), running in an oval fashion to cover three quarters of the cortical bone on the anterior side (under tension). Cigar shaped pores coming in from the periosteal surface were still visible, but not as large as those which were observed at 14 days of age. Cement lines were seen below the tide line only on the endosteal side and no evidence of secondary remodelling at the porous side of the cortex was present (Fig 3.9). In both strains, at either age, there was no cement lines seen at the porous (fast growing) side of the cortical bone indicating the absence of secondary remodelling.

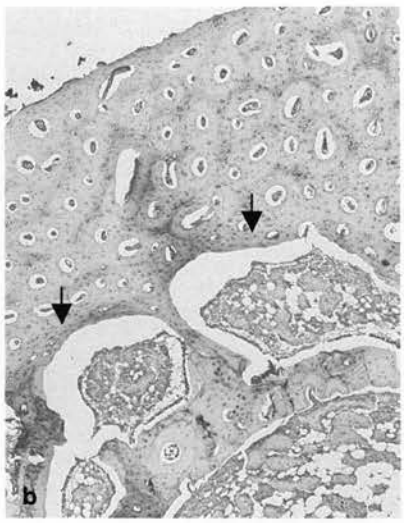


Figure 3.9 Secondary remodelling in 42 day old chickens within the (a) slow and (b) fast growing strains. The arrows indicate the tide line and secondary remodelling sites below it.

Confirmation that there was no resorption (secondary remodelling) at the periosteal surface was shown by identifying osteoclasts by TRAP staining. At both 14 and 42 days within both the fast and slow growing strains TRAP activity reacted strongly on the endosteal surfaces. However, there with no evidence of TRAP activity in the primary osteonal canals in the periosteal surface (Fig 3.10).

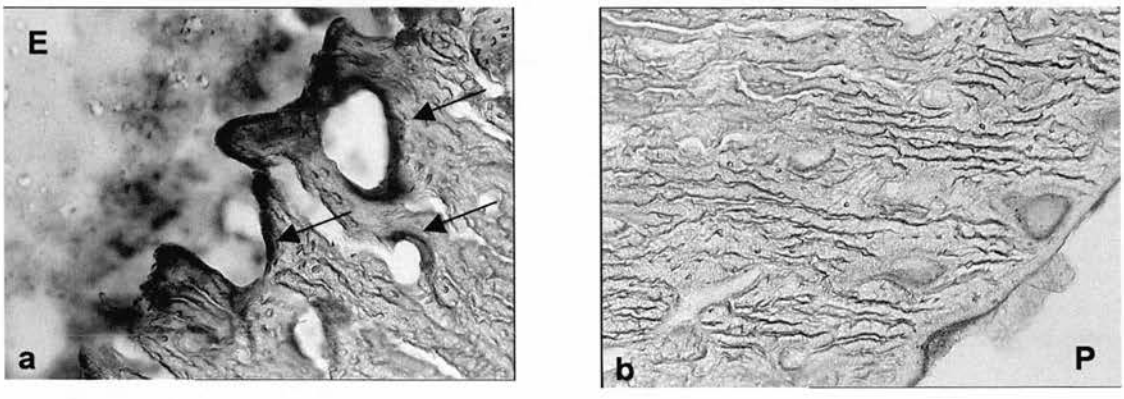


Figure 3.10 TRAP activity within a 42 day old fast growing chicken, at the endosteal surface (E) and within the canals at the endosteal surface (black arrows) (a), lack of TRAP activity at the periosteal surface (P) (b).

3.4.5: Primary osteon infilling

There was slower infilling of the primary osteons in the rapidly growing birds periosteally at 14 days of age (F = 20.8 ± 2.8 %; S = 55.8 ± 2.8%; P < 0.001) and at 42 days of age (F = 57.5 ± 3.0 %; S = 72.8 ± 3.0%; P < 0.001) (Fig 3.11).

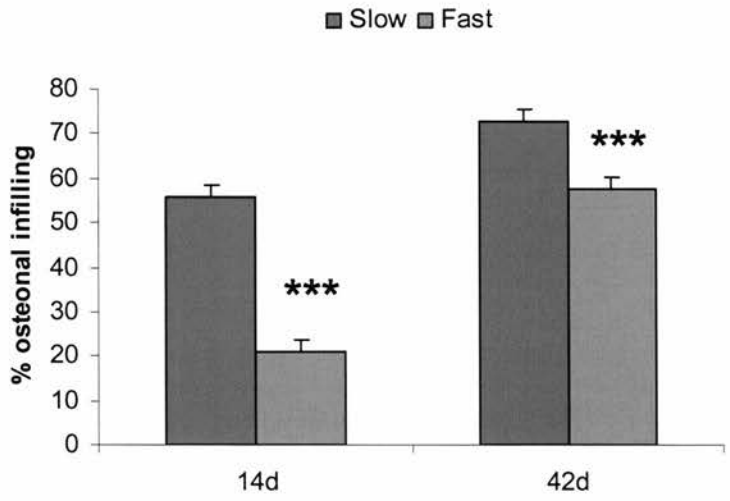


Figure 3.11 Primary osteonal infilling in fast (n=10) and slow (n=10) growing chickens at 14 and 42 days. Data are shown as mean ± SEM, ***P<0.001.

The decrease in % osteon infilling may be misleading as it could be due to similar infilling rates by the osteoblasts but in larger osteons of the fast growing birds. This combination would also result in a decreased % osteon infilling. Therefore to answer this question I measured the size of the osteonal area in the two strains at 14 days (F = 6703 ± 321.4 μm^2, S = 6341 ± 321.4μm^2) and 42 days (F = 5405 ± 321.4 μm^2, S = 4773 ± 321.4μm^2) (Fig 3.12).

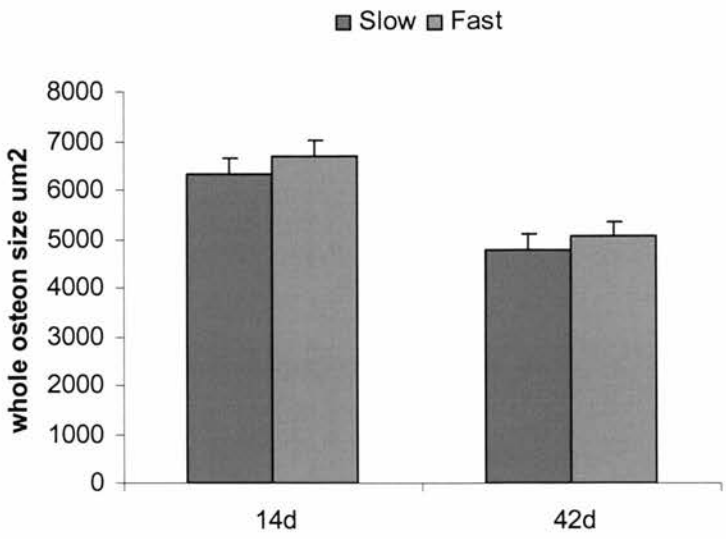
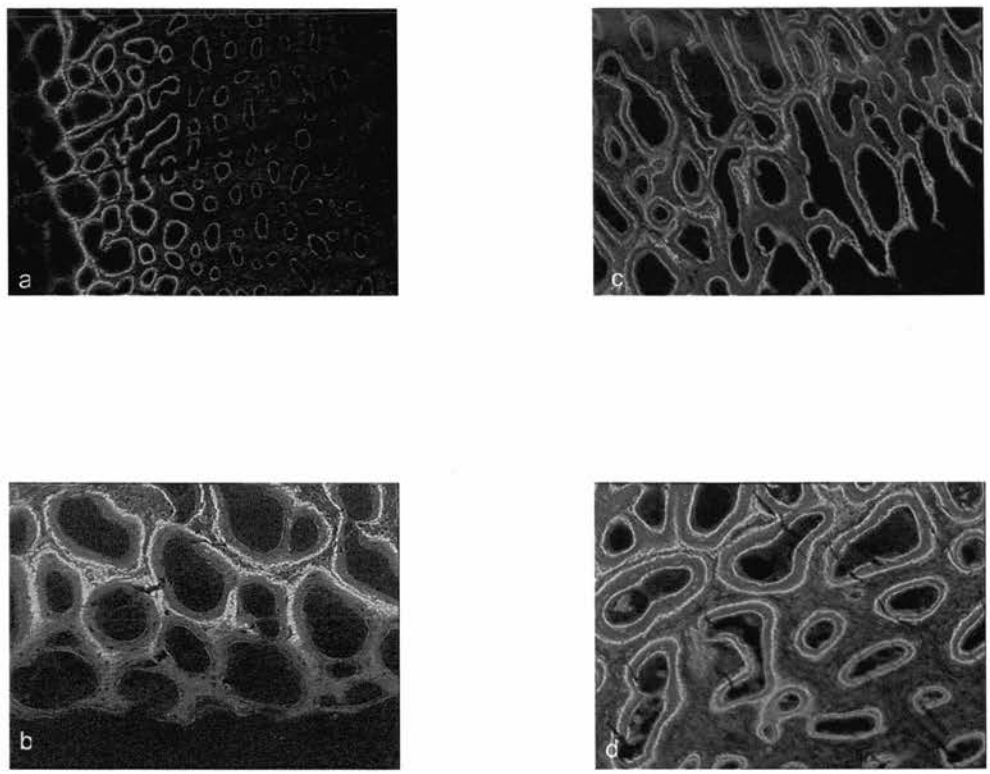


Figure 3.12 Osteonal size in fast (n=10) and slow (n=10) growing chickens at 14 and 42 days of age. Data are shown as mean ± SEM.

This indicated that there was no statistical difference in osteonal size between the two strains suggesting that the osteoblast activity within the osteons of the fast growing strain was less than that of the control birds.

3.4.6: Determination of optimal calcein labelling injection intervals.

Examination of injection schedule to determine MAR showed that double labels of calcein administered with a twenty-four and forty-eight hour interval showed only a single label within osteons at the periosteal edge. Administration with an interval of seventy-two hours interval, resulted in a double calcein label within the osteons at the periosteal edge (Fig 3.13). With an interval of ninety-six hours the first label was seen to be disappearing at certain points within the osteons closer to the endosteal edge. From this pilot study a time interval of 72 hours between labels was deemed optimal.



a 48 hrs between calcein labels, only a single label seen.
b 48 hrs between calcein labels, only a single label seen.
c 72 hrs between calcein labels, double label seen.
d 72 hrs between calcein labels, double label seen.

Figure 3.13 Time scale to optimise calcein labelling. (a+c) original magnification = x100. (b+d) original magnification = x500.

3.4.7: Mineral apposition rate

In order to directly assess osteoblast mediated osteonal infilling I assessed the MAR within the osteons of the periosteal surface at 21 days of age in birds of both strains. This clearly showed that the fast growing strain had a slower MAR than that of the slow growing strain (F = 11.51 ± 4.3μm/day, S = 28.16 ± 2.6μm/day; $P < 0.001$) (Fig 3.14).

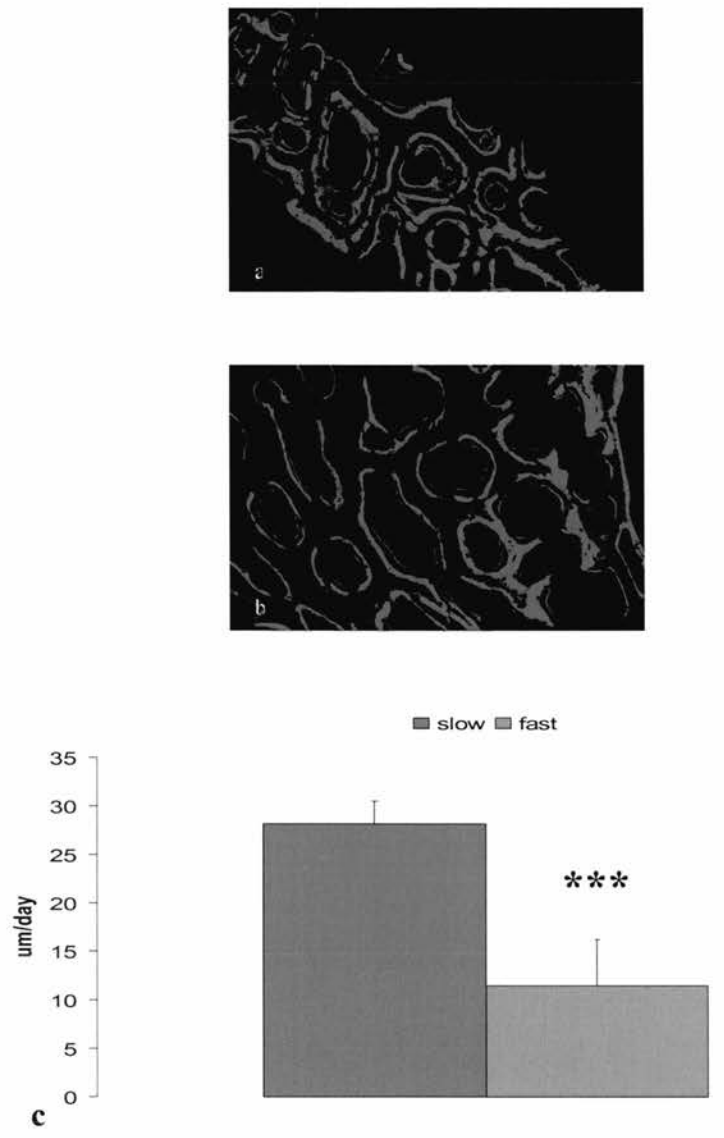


Figure 3.14 Calcein labelling at 21 days in (a) slow (n=10) and (b) fast growing birds (n=10) within the primary osteons of the periosteal region. A significant difference in mineral apposition rate was observed (c). Data are shown as mean ± SEM, ***P<0.001.

3.5: Discussion

In a former study it was reported that within fast and slow growing birds, periosteal porosity was increased in the faster growing birds (Williams et al 2000, 2004). This was confirmed by the findings of this study, where the fast growing birds were found to have a wider tibiotarsi but the cortex was more porous at the periosteal edge. In

addition, Williams and colleagues (2004) found that by feed restricting the fast growing strain, the tibiotarsi width was similar to that of a slow growing chicken and the porosity at the periosteal surface was also reduced to similar levels as the slow growing strain. This highlights the relationship between the rate of body growth and cortical bone porosity. Bone width is of great importance to bone strength (Schwartz and Biewener 1992) and as expected the fast growing strain had wider tibiotarsi than that of the control strain at both ages. It is therefore likely that in order to increase the bone loading capacity as quickly as possible, the fast growing strain expanded its tibiotarsi width more rapidly to compensate for the greater load placed upon it. By expanding rapidly by both periosteal apposition and production of new osteons at the periosteal surface, it is therefore likely that the newly formed primary osteons of the fast growing strain have less time to infill the new osteons by bone apposition before reaching the endosteal surface where they are reabsorbed by expansion of the marrow cavity.

As expected, the body weights showed the birds of the slow growing strain to be half the size of the birds of the fast growing strain (Fig 3.1). This confirms previously published results (Williams et al 2000a). All chickens were sourced from the same farm and kept under identical conditions, therefore the variance noted between farms in a previously reported study does not apply (Williams et al 2000a). According to my hypothesis, the greater body weight of the fast growing birds resulted in a load induced increase in primary bone formation at the periosteal surface. This response is similar to that observed in other growing animals; In young beagle dogs the bones of immobilised forelimbs developed at a reduced rate with a smaller cross-section than the bones of the normally loaded limb (Uhtoff and Jaworski 1978).

The tibiotarsi of the control strain had the narrowest, weakest and lowest stiffness at both ages as expected for the weight of the chicken. The greater bone thickness and stiffness in the heavier chickens prevents the bones bending under the weight of the chicken. In a healthy growing skeleton the bone mass and architecture alters to accommodate the load placed upon it. Early experiments on bone adaptation to loading were carried out on birds. Artificially imposing an intermitant load on a turkey ulna has been found to increase the cross-sectional area of the bone, primarily by widening the periosteal surface with new bone and primary osteon formation. In addition, it was found that the increase was proportional to the magnitude on the load placed upon the bone (Rubin and Lanyon 1987). This would apply to the fast growing strain of bird in this study. By walking around the pens in which they were kept, an intermitant load is placed upon the birds tibiotarsi, so that the heavier strain with the greater body weight had a greater load, and the larger expected bone area. However by removing the variable of the body weight it is possible to see that although the slow strain has thinner bones, the cortex has developed in a natural way rather than being forced to accommodate excess weight. The bones of the slow growing strain were predictably stronger.

The cortical width achieved by the fast growing strain was greater than that of the slow growing strain, but periosteally the cortical bone was consistently more porous in the fast chickens. In numerous adults species there is the constant remodelling of the bone around the Haversian canals of individual osteons. This maintains the skeletons mechanical competence and allows for its role in mineral homeostasis (Parfitt et al 1983, Frost 1986). In many cases porosity can be the imbalance of remodelling (Frost 1997). The increased porosity observed in the fast growing strain was not seen as a remodelling imbalance as the study of cement line staining (an

indication of secondary remodelling) showed no remodelling at the periosteal surface, where the increased porosity was noted. This implies that the bulk of bone is achieved by the simultaneous expansion in the tibia width and growth of the marrow cavity, rather, than by active cutting cones remodelling the periosteal surface. Confirmation that bone resorption was not taking place within the periosteal edge of the cortical was determined by TRAP staining. Enzyme activity was only shown to be a present endosteally within the cortical bone. It has been reported in two previous studies that periosteal porosity reduced with age (Williams et al 2000, Williams et al 2004). This is confirmed in this study. It has also been reported that growth restriction was found to significantly reduce porosity and this further highlights the correlation between the rate of body weight increase and cortical bone porosity (Williams et al 2004).

It has previously been suggested that the cause of increased porosity might be due to inadequate mineral supply in the feed given to the fast growing modern strain (Thorp and Waddington 1997). Reported low ash content (Williams et al 2000b) is consistant with mineral deficiency, but this could also be a problem caused by the fast growers being unable to utilise the mineral provided quickly enough to accommodate the rapid growth rate. The greater porosity could also simply be part of the phenotype of the fast strain as LeTerrier et al 1998 reported. By reducing the growth rate of the fast growing strain with energy restriction, LeTerrier and colleagues (1998) showed there was no reduction in cortical porosity even though there was a reduction in the chicken growth. These results are at variance with Williams et al 2004, who found that cortical porosity was reduced with growth restriction. However these differences between the two studies could be due to the smaller reduction of energy and protein content in the study of LeTerrier et al 1998.

It was possible that the growth restriction in the French study (LeTerrier et al 1998) was not large enough to produce a detectable effect. Williams and colleagues (2004) produced a growth restriction that resulted in stiffness and breaking strength values similar to the control strain of this study. Studies of embryonic chickens of different strains which included those of a fast growing meat-type, showed that enhanced growth reduced skeletal adaptability. This led the authors to suggest that high growth rates resulted in bones having relative insensitivity to loading (Pitsillides et al 1999).

As there was no evidence that the increased porosity was due to greater osteonal resorption it was possible the fault was due to inadequate osteonal infilling. During the infilling of primary osteons, bone formation occurs in two distinct stages; firstly the matrix formation by the osteoblasts, followed by its mineralisation. Both events are seperated in time and space (Recker 1983). As the primary osteon sizes were not different between the two strains, the measurement of the amount of bone laid down by the osteoblasts, within the newly formed osteons, was a necessity. The amount of bone laid down within the periosteal primary osteons was lowered in the fast growing strain.

The MAR studies confirmed that cortical porosity was due to the lack of infilling within the primary osteons at the periosteal edge. MAR has been previously reported in growing broilers to an average of 13.4 μm/day (Newbrey et al 1988) which is similar to the growth rate of the fast growing chickens in this study. In comparison the MAR within the primary osteons at the periosteal surface of the slow growing chicken was more that double this amount.

In the growing long bone such as the tibiotarsus, there are three distinct compartments contributing to the growth and strength of the shaft (Frost 1973). In relation to this study the main compartment of interest is the outer periosteal

compartment, which is directed towards osteoid production and mineralisation. The broilers in Newbrey's study (1995) showed an extremely active periosteal surface, again similar to those of the fast growing strain of this study. It can therefore be speculated that the fast growing strain failed to respond to the mechanical stimulation with bone differentiation failing to keep pace with the skeletal support requirements. The widening of the cortex is an attempt by the bird to adapt to the rapidly increasing body weight. However the increased porosity is an indication that there is an intrinsic failing within the bone to adapt. Possible explanations for the impairment of osteonal infilling may include: (1) a reduced number of osteoblasts incorporated into the primary osteon capable of infilling the resultant canal and (2); the incorporated osteoblasts are incapable of laying down the required amount to fill in the resultant canal. Either scenario would result in a lowered infilling of the canal.

3.6: Conclusions

Under identical conditions the two strains of chickens showed many similarities in the development of their tibiotarsi. Both continually increased the width of their tibiotarsi to support their growing body weights. However the quality of bone produced by the fast strain was relatively poor in terms of porosity which probably accounts for the observed lowered effective breaking strength observed in these chickens. The cause of the porosity has been shown to be due to a lack of infilling within the newly formed primary osteons.

Chapter 4: Determination of the cause of decreased osteonal infilling in the fast growing broiler. A histomorphometric and immunocytochemical study

4.1: Introduction

Cell proliferation within the periosteum and the production of osteoblasts is necessary for cortical bone to increase in size periosteally and also to make osteoblasts available for incorporation into the newly forming primary osteons (Doty and Schofield 1976). The periosteum is described as an envelope that covers the bone surface and separates it from surrounding soft tissue. It is highly adherent to the epiphysis and slightly less adherent to the bone surface in the diaphyseal region (Kitaoka et al 1998). It is made up of two layers, an outer fibrous layer and an inner cellular cambian layer (Ragsdale et al 1981, Balena et al 1992). Within the growing skeleton the periosteum is highly osteogenic, with epithelial like cells, nerve fibres and blood vessels found in the cambian layer and fibroblast like cells found in the outer fibrous layer (Uchida et al 1988). In an adult human there is minimal cellular activity in the cambian layer (fibrous layer) (Eyre-Brooke 1984), although it does show a higher osteogenic potential in response to pathologic stimuli (Budal 1979). It is possible that a lower osteoblast and osteoblast progenitor proliferation within the fast growing strain of chickens could limit the amount of osteoblasts available for incorporation into primary osteons. Such a series of events may account for the lack of osteon infilling and increased porosity in the fast growing chickens.

Blood vessels from the periosteum are incorporated in the newly formed primary osteons. Longitudinal depressions form at the periosteal surface at the areas where blood vessels sit. These then become covered by more bone and thereby trapping the blood vessel within the bone. The resultant hollow cylinder contains blood vessels, nerves and osteoblasts which synthesise bone and form rings of boney lamellae within the hollow. This results in the formation of the circular primary osteons (Banks 1986). It is unclear if the number of blood vessels on the

periosteal surface influences the number and distribution of osteons formed. It is possible that differences in blood vessel number on the periosteal surface may account for, in some way, the altered osteonal infilling rates noted in the fast growing birds.

4.2: Aims

To determine the underlying cellular mechanisms that may be responsible for the lack of infilling of primary osteons observed in the fast growing strain of chicken. Specifically to study (a) the blood vessel number and (b) the cell proliferation within the periosteum, (c) the collagen orientation and osteocyte density within the fast and slow growing birds.

4.3: Materials and methods

4.3.1: Blood vessel staining

Determination of effective antibody staining to basement membrane proteins.

In order to determine optimum procedures for the detection of basement membranes, frozen sections of chicken liver and heart (rich in basement membranes), were tested with three primary antibodies to basement membrane constituents as described in chapter 2 (section 2.1.12).

Birds and Housing

At 21 days of age, 3 birds from each brooder in study 1 (section 2.1.1) (6 birds of each strain in total) were picked at random. The birds were killed by cervical

dislocation and sections of the right tibiotarsus were processed for examination in this study.

4.3.2: Bromo deoxyuridine Labelling

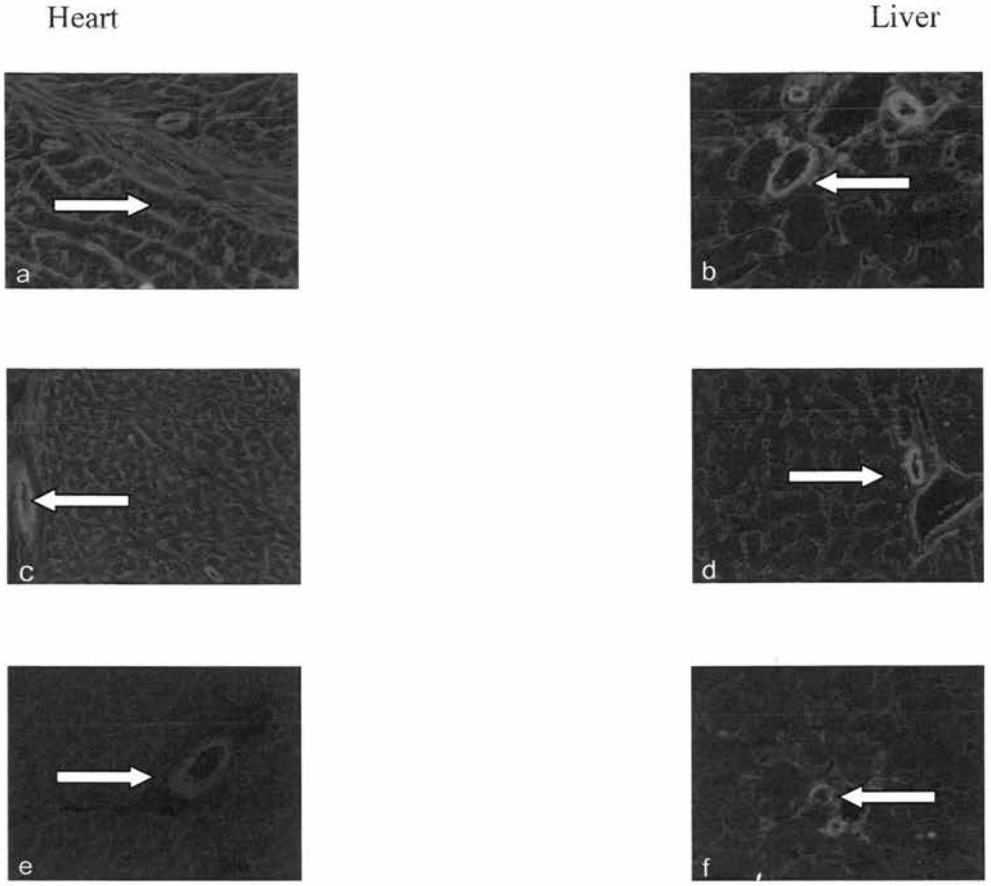
Birds and housing

At 20 days of age, 6 birds from each brooder in study 1 (section 2.1.1) (12 birds of each strain in total) were picked at random, intravenously injected with BrdU at four time points between 12 and 24 hours before cull. The birds were killed by cervical dislocation and sections of the right tibiotarsus were processed as described in chapter 2 (section 2.1.6).

4.4: Results

4.4.1: Basement membrane staining in frozen sections of chick heart and liver

In pilot studies, frozen sections were studied to determine which antibodies to basement membrane proteins gave clear results on chicken tissue. All primary antibodies; laminin (31 and 3H11) and heparan sulphate (33) detected basement membrane in both tissues. Control sections were negative. The immunostaining with antibodies 3H11 was stronger and superior to that obtained by staining with antibody of 33. Both were superior to antibody 31 (Fig 4.1).



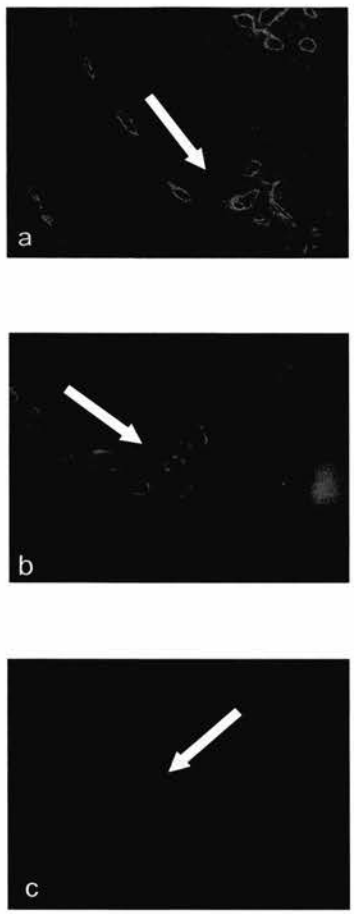
a Ab 3H11 on chick heart tissue.
b Ab 3H11 on chick liver tissue
c Ab 33 on chick heart tissue
d Ab 33 on chick liver tissue
e Ab 31 on chick heart tissue
f Ab 31 on chick liver tissue

Figure 4.1 Basement membrane staining of chicken heart and liver. All original magnifications were at x100. Arrows indicate the strongest areas of basement membrane staining.

4.4.2: Demasking wax embedded slides

Trypsin 3pk pre-treatment gave negative results with all of the basement membrane antibodies. Proteinase K and pronase pre-treatment gave positive results with the laminin antibody 3H11 but neither methods worked with the heparan sulphate antibody 33 (Fig 4.2). Therefore, all further work was carried

out on decalcified paraffin embedded sections, pretreated with proteinase K, as detailed in chapter 2 (section 2.1.5.3).



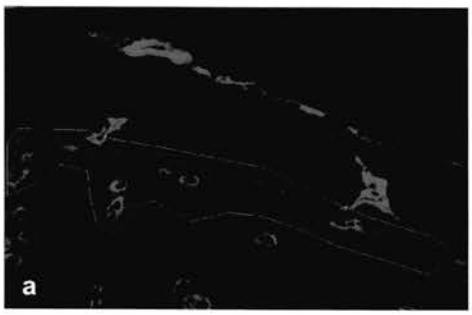
a proteinase K demasking with antibody 3H11
b pronase demasking with antibody 3H11
c trypsin 3pk demasking with antibody 3H11

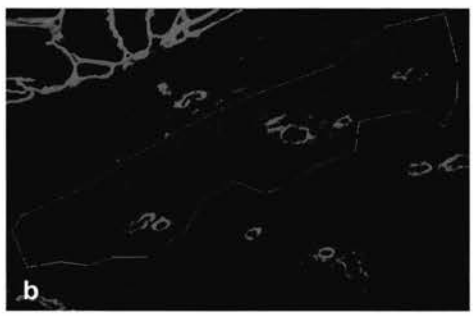
Figure 4.2 Demasking methods for wax embedded sections for use with antibodies to basement membrane proteins.

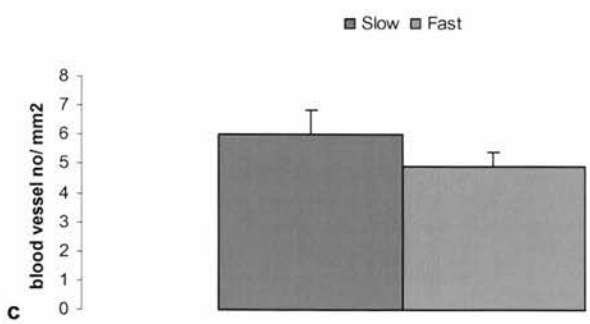
4.4.3: Blood Vessel number within the Periosteum

Blood vessel number within the periosteum was similar in both strains (F = 6.0 ± 0.5 /mm^2, S = 4.91 ± 0.7 /mm^2 ;NS) but differed between regions habitually loaded in tension (anterior: 3.82 ± 0.2 /mm^2 [fast growth area]) or in compression

(posterior 6.14 ± 1.3/mm^2; P<0.01 [slow growth area]) in both strains of chicken. However there was no significant strain/area interactions in the number of blood vessels available in the periosteum (Fig 4.3).







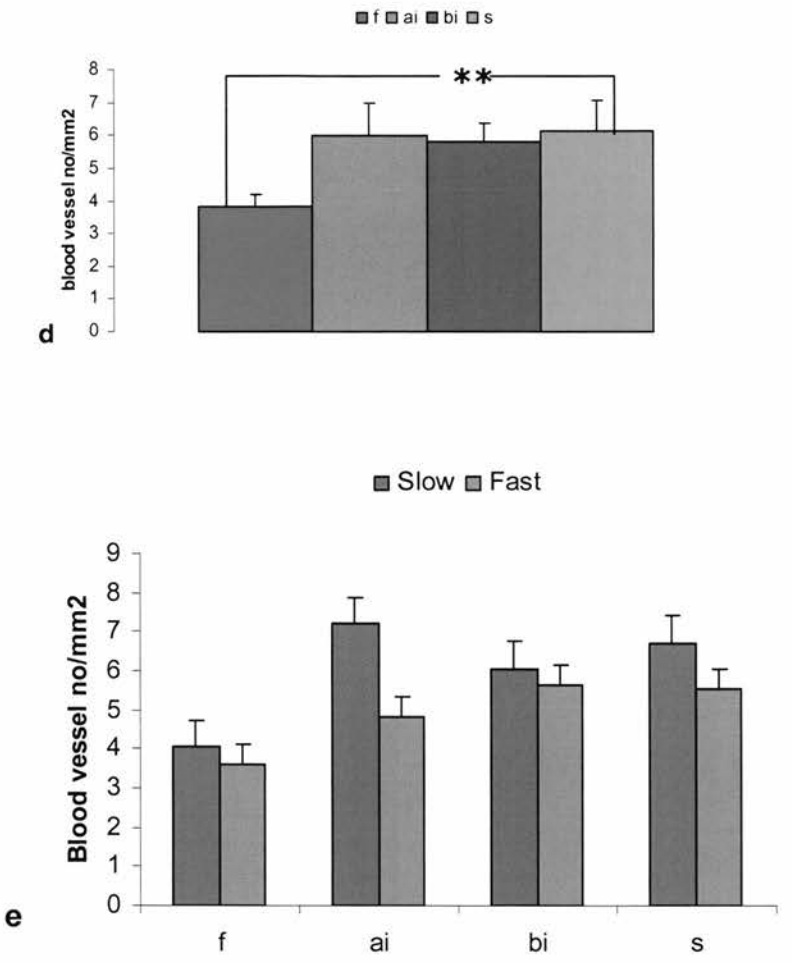
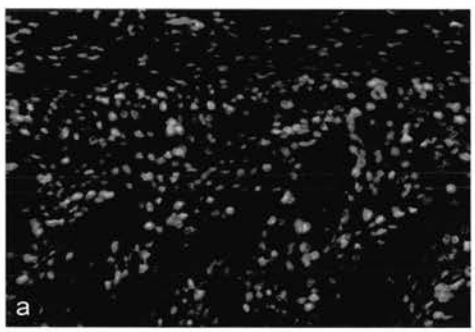


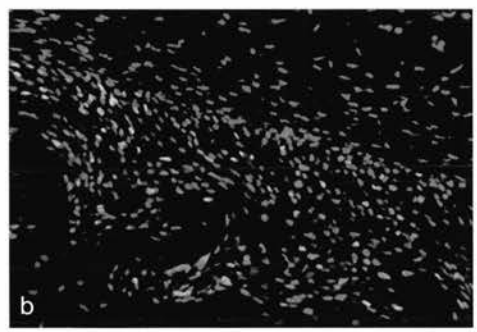
Figure 4.3 Blood vessel number in slow (n=6)(a) and fast (n=6)(b) growing chickens, (the boxed off area is the inner layer of the periosteum where blood vessels were counted). The difference in the number of blood vessels between strains (c), between different areas of the bone (d), and strain/area interaction (e). (Where f is the fast growing area of the periosteum, s is the slow growing area of the periosteum, and ai and bi being the intermediate areas between the fast and slow growing areas). (The fast area is the anterior side; under tension, the slow area is the posterior side; under compression). Data are shown as mean ± SEM, ** $P<0.01$.

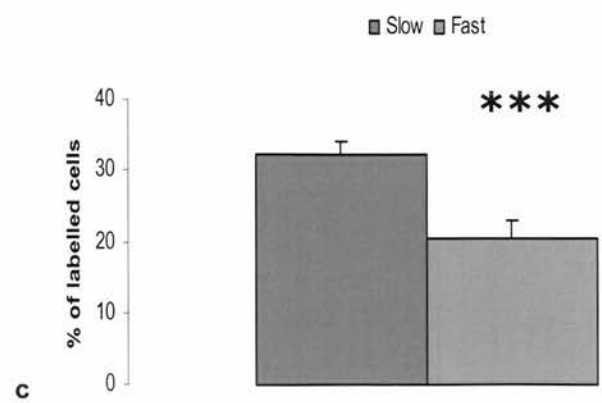
4.4.4: Cell proliferation in the periosteum

Proliferating pre-osteoblasts (Fig 4.4a and b) within the osteogenic layer of the periosteum had a lower labelling index in the rapidly growing birds averaged across the four circumferential areas of the periosteum (F = 20.47 ± 2.6 %, S =

32.17 ± 2.2 %; P<0.001) (Fig 4.4c). Comparison of the labelling index between the areas of the bone under fast growth (anterior) when compared to areas under slow growth (posterior) indicated that there was a significant difference between these two areas with the fast growth area having a higher labelling index than that of the slow growth area (fast growth area = 38.99 ± 0.6 %, slow growth area = 17.43 ± 1.6 %, P <0.001) (Fig 4.4d). There were varying significant differences when strain/area interactions were considered ([fast growth area = F = 29.96 ± 2.6 %; S = 48.92 ± 1.8 %]; [intermediate area a = F = 19.05 ± 6 %, S = 31.04 ± 1.6 %]; [intermediate area b = F = 18.43 ± 2.2 %, S = 28 ± 1.7 %,]; [slow growth area = F = 14.45 ± 2.0 %, S = 20.7 ± 2.2 %]) with interactions as (f-ai = P< 0.05; f-bi = P< 0.01; f-s = P< 0.001) when compared to the fastest growing area (Student's t test) (Fig 4.4e).







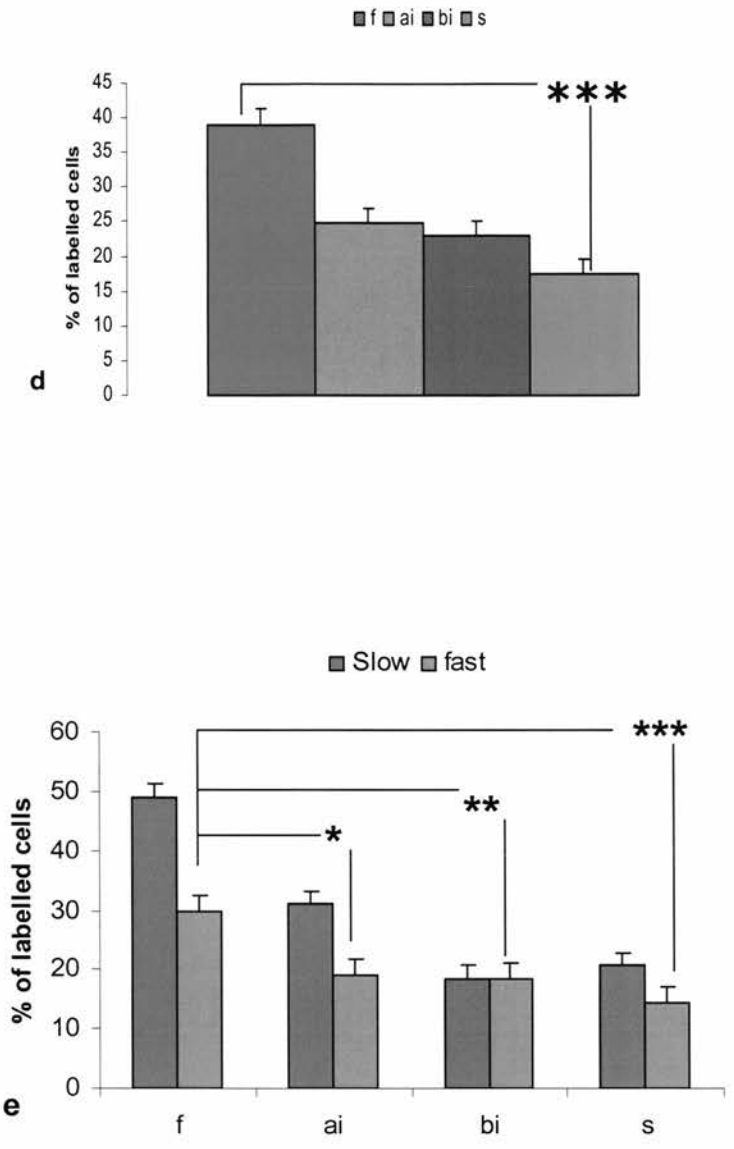
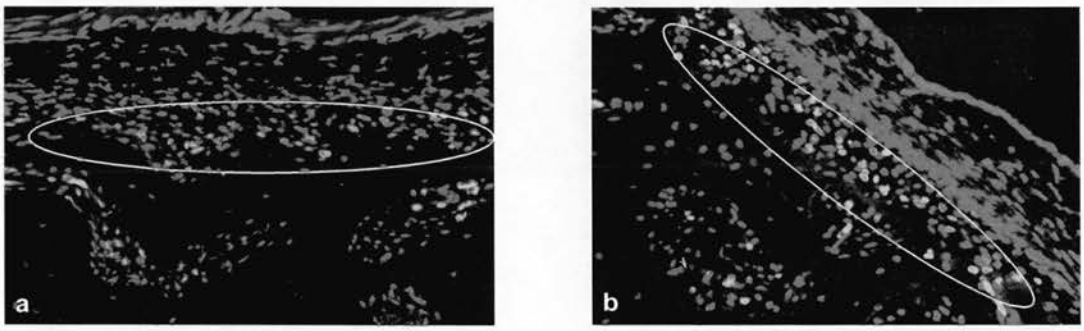
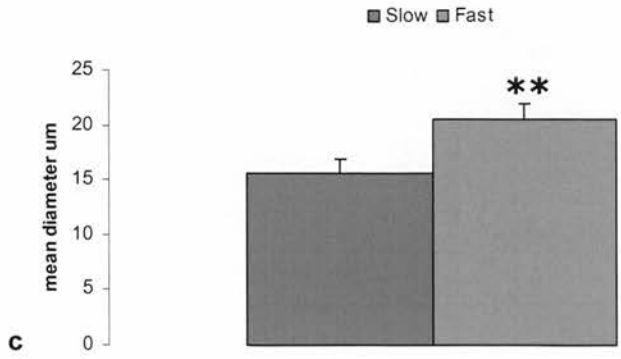


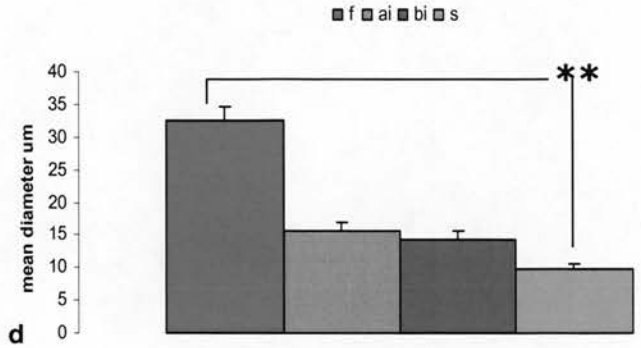
Figure 4.4 Labelling index of cells within the periosteum in slow (n=6)(a) and fast (n=6)(b) growing chickens. The difference in the labelling index between strains (c), between different areas of the bone (d), and strain/area interaction (e). (Where f is the fast growing area of the periosteum, s the slow growing area of the periosteum, and ai and bi being the intermediate areas between the fast and slow growing areas). (The fast area is the anterior side; under tension, the slow areas is the posterior side; under compression). Data are shown as mean ± SEM, * $P<0.05$; ** $P<0.01$; ***$P<0.001$.

4.4.5: Periosteum diameter

The osteogenic layer of the periosteum (Fig 4.5a and b) had a greater diameter in the fast strain of chickens (F = 20.57 ± 1.9 μm, S = 15.54 ± 1.7 μm; P<0.01) (Fig 4.5c). There was also a clear difference in the size of the osteogenic layer between the fast growing side and the slow growing side of the bones (fast area = 32.56 ± 2.0 μm^2, slow area = 9.71 ± 1.8 μm^2; P<0.01) (Fig 4.5d). However there was no significant strain/area interactions in the size of the osteogenic layer of the periosteum (Fig 4.5e).







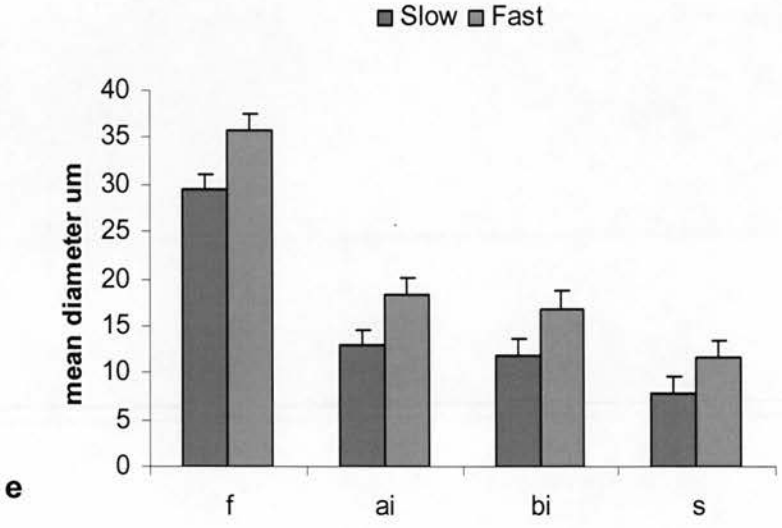


Figure 4.5 Periosteum diameter in slow (n=6)(a) and fast (n=6)(b) growing chickens, (the boxed off area is the inner layer of the periosteum where the proliferating cells are found). The difference in the periosteum width between strains (c), between different areas of the periosteum (d), and strain/area interaction (e). (Where f is the fast growing area of the periosteum, s the slow growing area of the periosteum, and ai and bi being the intermediate areas between the fast and slow growing areas). (The fast area is the anterior side; under tension, the slow areas is the posterior side; under compression). Data are shown as mean ± SEM, ** $P<0.01$.

4.4.6: Live/dead cell detection

It was considered that the osteoblast live/dead ratio within infilling osteons may explain the difference in infilling rates of the primary osteons at the periosteal edge between the fast and slow strains. Live/dead detection was carried out to determine the ratio of live cells to dead cells by the penetration of two cell markers. One marks live cells green and one marks dead cells red. This was used to determine if there was any difference between the two strains in the number of osteoblasts which may have undergone apoptosis. Unfortunately the probes did not penetrate into the undecalcified bone after submersion in the probes for 5 or 24 hours. This method has been shown by another group to penetrate into undecalcified growth plate blocks of tissue (H Roach, unpublished observations). This line of investigation was not pursued.

4.4.7: Osteocyte density

Whilst studying slides to determine infilling rates of primary osteons (chapter 3) I made a serendipitous observation. It appeared that the osteocyte density in the bone of the fast growing birds was greater than that of the slow growing birds. This observation may reflect differences in osteoblast differentiation rates between the two strains and was therefore investigated further. Osteocyte density within the circumferential lamellae at the periosteal edge was higher within the rapidly growing birds at 14 days of age (F = 5.25 ± 0.0004/mm^2, S = 3.56, ± 0.0004/mm^2; P , 0.01) and at 42 days of age (F = 5.34 ± 0.0003/mm^2, S = 1.8 ± 0.0003 mm^2; P , 0.01) (Fig 4.6). However osteocyte density was unchanged within the newly laid down bone of the primary osteons at 14 days of age (F = 4.47 ± 0.04 mm^2, S = 3.86 ± 0.03 mm^2) and 42 days of age (F = 2.72 ± 0.048 mm^2, S = 2.85 ± 0.032 mm^2) (Fig 4.7).

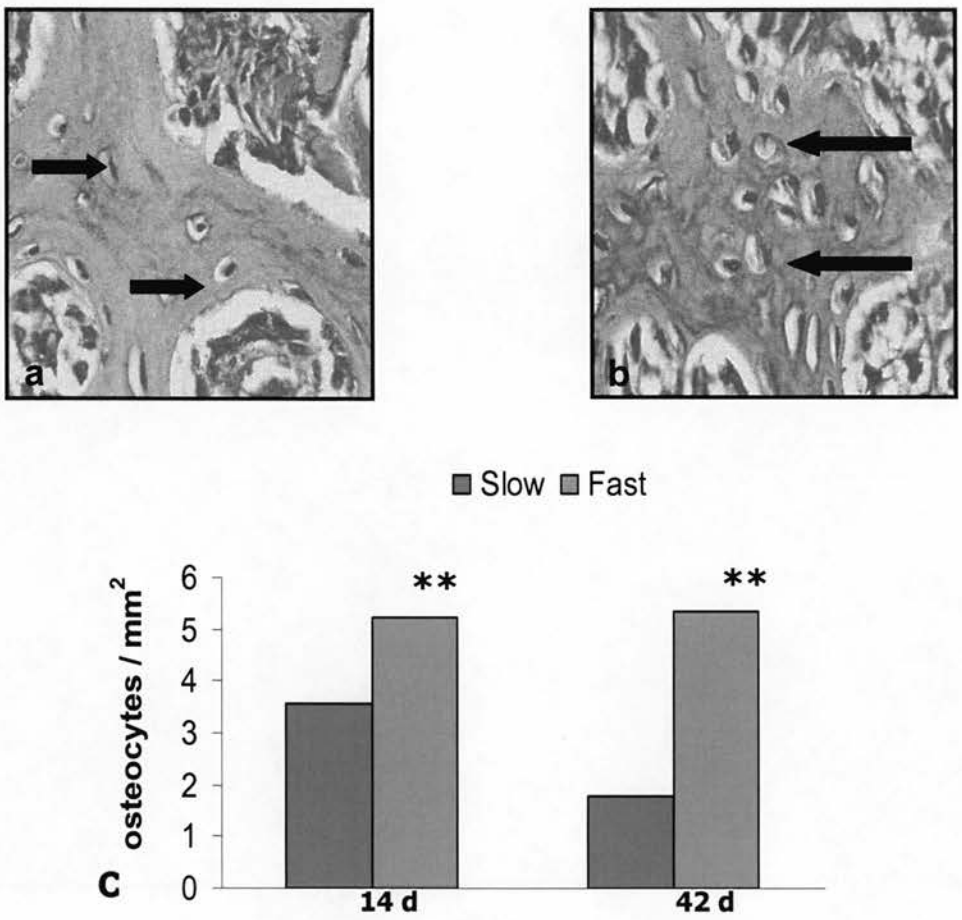
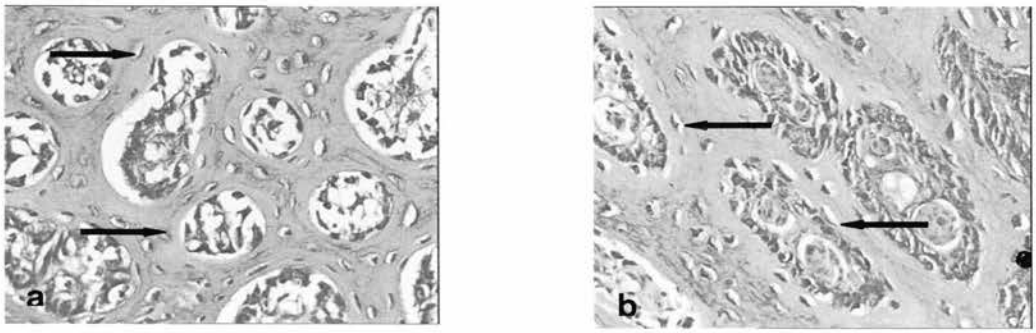
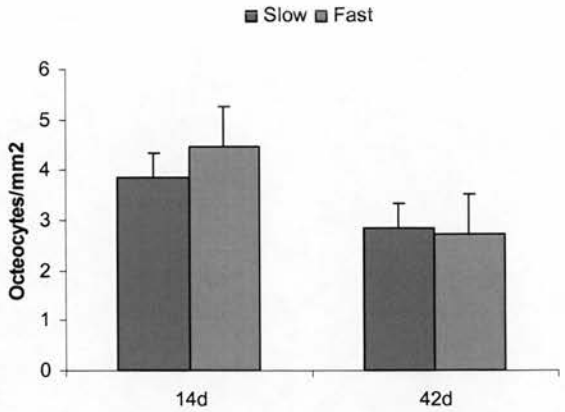


Figure 4.6 Osteocyte density within the circumferential lamellae at the periosteal edge of both slow (n=10)(a), and fast (n=10)(b) strains of chicken at 14 and 42 days of age. Quantification of osteocyte density (c). Error bars were too small to be visible on the graph. Data are shown as mean ± SEM, ** $P<0.01$.



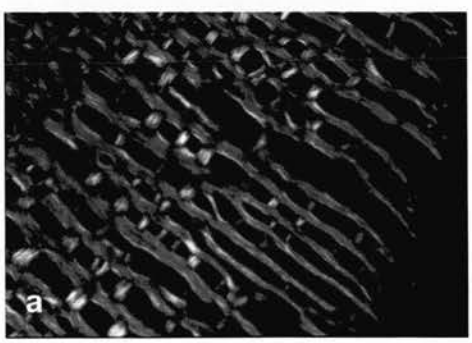


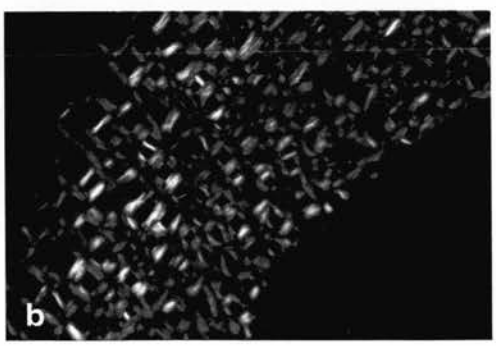
C

Figure 4.7 Osteocyte density within newly formed primary osteons at the periosteal edge of both slow (n=10)(a), and fast (n=10)(b) strains of chick at 14 and 42 days of age. Quantification of osteocyte density (c). Data are shown as mean ± SEM.

4.4.8: Collagen orientation

Collagen orientation at the periosteal edge of both the fast and slow growth strain of chickens showed a circumferential collagen orientation within the lamellae of the newly formed osteons. There was more alternating collagen fibre orientation within the circumferential bone between the osteons than in the osteons themselves (Fig 4.8). There was no obvious visual differences in collagen orientation between the two strains at either the anterior or posterior areas of the cortex.





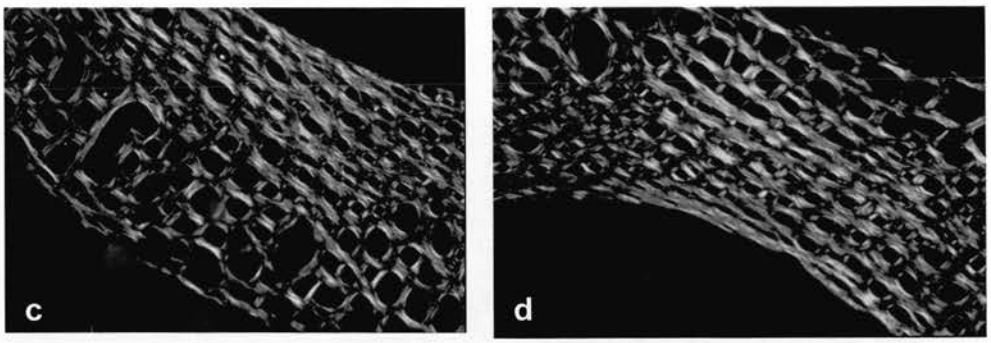


Figure 4.8 Collagen fibre orientation observed with polarised light, at the fastest growing area of the cortical bone in both fast (n=6)(a) and slow (n=6)(c) growing strains. At the slowest growing area of the cortical bone in both fast (b) and slow (d) strains. Original magnification was at X100.

4.5: Discussion

Blood vessel numbers were found not to be a determinant for primary osteon porosity, with both strains of chicken having almost the same amount of blood vessels available for incorporation at all times. However differences were noted between different anatomical areas of the bone. The anterior, which is always under tension, had a reduced number of blood vessels in both strains compared to that of the posterior, which is always under compression. This indicates that the area which is growing fastest around the cortex of the bone had fewer blood vessel available for incorporation than the side which was growing slowly. This indicates that blood vessel number is affected by the way in which the mechanical load (body weight) is applied to the bone but not by the strain and growth rates of the chickens. This rules out the hypothesis that blood vessel availability at the periosteum for incorporation into primary osteons can effect the porosity seen at the periosteal edge. Previous studies into cell proliferation within the avian species have concentrated on cell proliferation within the growth plates and

correlations have been made between chondrocyte proliferation and growth rate of the bones (Kirkwood et al 1989). BrdU has been successfully used as an indicator of proliferating cells in rat growth plates and neural tissue (Harms et al 1986, Farquharson and Loveridge 1990, Kee et el 2002) however no published data on cell proliferation rates within the periosteum of chicken bones exist. It is the inner layer of the periosteum (cambium) which contain the proliferating cells (Balena et al 1992) and periosteal osteoblasts are presumably derived from precursor cells that reside in the cambium layer adjacent to the bone surface (Ellender et al 1988, Wlodarski 1989, Squier et al 1990). The lower labelling index within the periosteum of the fast growing strain indicates that less cells were proliferating even though a higher cell number existed. The higher cell number may be related to the fact that the periosteum was wider in the fast growing strain compared to that of the slow growing strain. Periosteum width was also related to whether the bone was under tension or compression but this was not affected by the strain of bird. The lower labelling index at the periosteum and the increased osteocyte density within the circumferential lamellae of the fast growing strain suggests an increase in differentiation and transit time through the osteoblast lineage at the periosteal surface. This hypothesis could also account for the reduced primary osteon infilling, but osteocyte density within primary osteons was similar in both strains. It is well documented that increased osteocyte apoptosis is correlated to sites of bone fracture (Noble et el 1998), however apoptotic rates in osteoblasts of infilling osteons are not known. Osteoblasts are responsible for the formation of osteoid (Owen 1963), and are specific to sites where bone apposition is taking place (Thomson and Loveridge 1992). The fast growing chicken increases its periosteal width in proportion to body weight and therefore it would appear that there are enough osteoblasts on the cortical surface to enable this expansion to

occur. It therefore is possible that the osteoblasts are pre-occupied by laying down new bone at the periosteal surface rather than proliferating. This may result in the number of osteoblasts incorporated into the primary osteons being limited within the fast growing strain.

Osteocytes are considered the most mature or terminally differientiated cell of the osteoblast lineage. They are osteoblasts which have been embedded in their own secretory products, occupying spaces, lacunae, in the interior of the bone. Molecular strain plays an important part in the modelling and remodelling of bone, and the best situated cell to detect the strain is the osteocyte (Lanyon 1993). As the mechanical pressure displaces the extracellular fluid, the surrounding matrix, including the osteocytes, are distorted (Turner et al 1994). Periosteal circumferential lamellae/bone of the fast growing birds had a higher osteocyte density. This may result from a faster rate of osteoblast differentiation in the fast growing birds. If the osteoblast transits through the matrix synthesising phase faster, each cell would be surrounded by less matrix and the resulting density of the future osteocytes would increase. When osteoblast precursors are locally available, as in the periosteum, this would have little or no effect on bone growth, as discussed above. However, if precursors were less readily available, as in the primary osteons, then this could result in reduced osteonal infilling due to the lack of osteoid. However, the osteocyte density within the new osteoid laid down in the primary osteons was the same between both strains, indicating that osteoblasts differentiate within the primary osteon at a similar rate in both strains. This suggest other mechanisms such as reduced osteoblast incorporation into primary osteons and/or increased apoptotic rates may be responsible.

To determine if cell death (apoptosis) was a cause of increased porosity within the periosteal primary osteons of the fast growing chickens, molecular probes were

used to detect live/dead cells. Unfortunately penetration of the probes into calcified bone blocks was unsuccessful. The problem was seen at both time periods (5 and 24 hours) studied. These probes have been shown to penetrate growth plates and their cells, with the CMFDA marking live cells green and EthD-1 marking dead cells red (Roach, personal communication). Should this be attempted again in further studies, it would be advisable to try the same probes on thinner blocks. Due to time, cost restraints and chicken availability this experiment was not repeated.

Given a particular porosity and mineralisation, and a particular trabecular or osteonal architecture, it remains possible for two regions of bone to have different material properties (Martin et al 1998). Therefore, the next variable to be considered was the orientation of constituent collagen fibres, which is controlled by both cell function and by extracellular physical processes known as micromodelling (Frost 1986). It has been shown that individual osteons as well as whole bone has collagen fibre orientation (Ascenzi and Bonucci 1967) that varies with position relative to the neutral axis. It has been shown that regions in habitual tension have more longitudinal fibres and those in compression have fewer (Portigiatti-Bardos et al 1983). In cortical bone, collagen fibre orientation may be the predominant factor in determining tensile strength (Martin and Ishida 1989). These authors however based this observation on cortical bone which generally shows little variance in porosity or mineralisation. Lamellar bone is stronger than woven bone in which the collagen fibres are more or less randomly orientated. The predominant orientation of collagen fibres affects tissue strength in relation to the mode of loading; longitudinal fibres promote strength in tension, while transverse fibres are associated with strength in compression (Martin et al 1998). In studies with cortical bone from bovine tibia, collagen fibre orientation was

considered a high predictor of bending properties. Density and mineralisation were considered the next best predictors. Variations in bending properties of plexiform and osteonal bone have been noted. In osteonal specimens 88% of the elastic modulus variability was accounted for by bone ash content and collagen fibre orientation (Martin and Ishida 1989, Martin and Boardman 1993). This present study defined the collagen fibre orientation according to Ascenzi and Bonucci 1967. No differences between the two strains was found. This indicates that the cause of tibiotarsi weakness in the fast growing strain was unlikely to be due to random orientation of collagen fibres. It was hypothesied that due to the high level of osteocytes and the rapid circumferential expansion of the tibiotarsi within the fast strain, there would be little collagen fibres organisation seen (Giraud-Guille 1988). It was more likely that the fibres would be laid down randomly and hence less visible by polarised microscopy. However this was not the case, with the fibres in both the osteon and the circumferential lamellae area having strong circumferential orientation. This indicates that although growth is rapid, the lamellae formation is still organised.

It is widely held that woven bone contains a greater cellular content (osteocytes) than typical cortical bone (Buckwalter et al 1995). Differences of 400-800% have been reported in osteocyte number between woven and lamellar bone. However after the assessment of collagen fibre orientation on this present study, it is clear that woven bone is not produced in any significant quantity in either of the strains examined in this study. Interestingly, woven bone formed at the periosteal surface in response to overload was similar in osteocyte number to normal periosteal cortical lamellae bone (Hernandez et al 2004). Only woven bone formed via endochondral ossification showed a significant osteocyte density increase (Hernandez et al 2004). These data indicate that the increased osteocyte density in

the lamellar bone of the fast growing strain is not a consequence of the presence of woven bone. The question remains as to what is the cause of the increased osteocyte density. Whatever the specific mechanisms are, it could be considered that the concentration of relevant signalling factors in bone may be increased in the bone tissue with a high cell density.

Skeletal adaptations vary in animals, in particular, changes in adaptive responses during growth. Cells can sense changes in their mechanical environment and promote alterations and adaptations in tissue structure and function. Mechanical stimuli regulate such fundamental processes as cell division and differentiation and determine tissue form (Benjamin and Hillen 2003). Development and growth of bones formed via a cartilage model involves two distinct patterns of bone formation. Endochondral bone formation, whereby cartilage matrix in the interior of growing metaphyses calcifies providing minerlised cores onto which recruited osteoblasts deposit new bone. In contrast perichondral bone formation is the process whereby bone is formed outside cartilage leading to the formation of a primitive cortex and associated periosteum (Riminucci et al 1998). When comparing immature to adult rats and their skeletal response to loading, Jarvinen et al 2003 concluded that young skeletons adapted through geometrical changes (increase in bone size), whereas adult rats seemed to adapt mainly through increase in bone density. The data from the fast and slow growing chickens of this study is in agreement with this view as both increased tibiotarsi width, with the fast increasing more rapidly with the greater load due to body weight being placed upon it.

4.6: Conclusion

In conclusion the data from this study indicates that the periosteal cortical porosity and the lack of infilling within the primary osteons (chapter 3, section 3.5.4) was not caused by blood vessel availability for incorporation into the osteons. Differences in collagen fibre orientation can also been ruled out and woven bone was not present. Osteocyte density in the circumferential lamellae increased in the fast strain but was similar in the primary osteons of both strains. These results indicate that the differences observed in osteon infilling between the strains is possibly due to a lack of availability of osteoblasts precursors at the periosteal surface. An increase in apoptosis of osteoblasts within the osteons of the fast growing birds cannot however be ruled out.

Chapter 5: Comparison of proliferation, differentiation and gene expression of osteoblasts: *In Vitro* Studies

5.1: Introduction

Bone formation takes place in animals not only during embryonic development and growth but throughout life in the process of normal bone remodelling and fracture repair. For both the growing skeleton and bone renewal throughout life there is the requirement for proliferation and differentiation of osteoprogenitor cells into osteoblasts. Unique properties and definition of the characteristic responses of the osteoblasts and mechanisms that control progression through the osteoblast cell linage allow study of any abnormalities in the skeletal development. Using primary cell cultures, the expression of cell growth and tissue specific genes during progressive establishment of the osteoblast phenotype allows the study of temporal developmental stages, in the osteoblast lineage.

Four principle osteoblast development stages can be defined (Stein and Lian 1995), 1) Proliferation supports expansion of the osteoblast cell population to form a multi-layered cellular nodule and biosynthesis of collagen type I. During this period genes necessary for activation of proliferation and cell cycle progression are expressed together with genes encoding growth factors. 2) Following proliferation, expression of genes associated with maturation and organisation are upregulated, rendering the extracellular matrix competent for mineralisation (Gerstenfeld et al 1993). 3) This is the development period which involves gene expression related to the accumulation of hydroxyapatite in the extracellular matrix. Genes encoding several proteins with mineral binding properties such as osteopontin, osteocalcin and bone sialoprotein expression, exhibit maximal expression at this time (Lian and Stein 1996). 4) A further development period follows in mature cultures during which collagenase is

elevated, apoptotic activity occurs and compensatory proliferation activity and collagen expression is evident (Lynch et al 1994) (Fig 5.1).

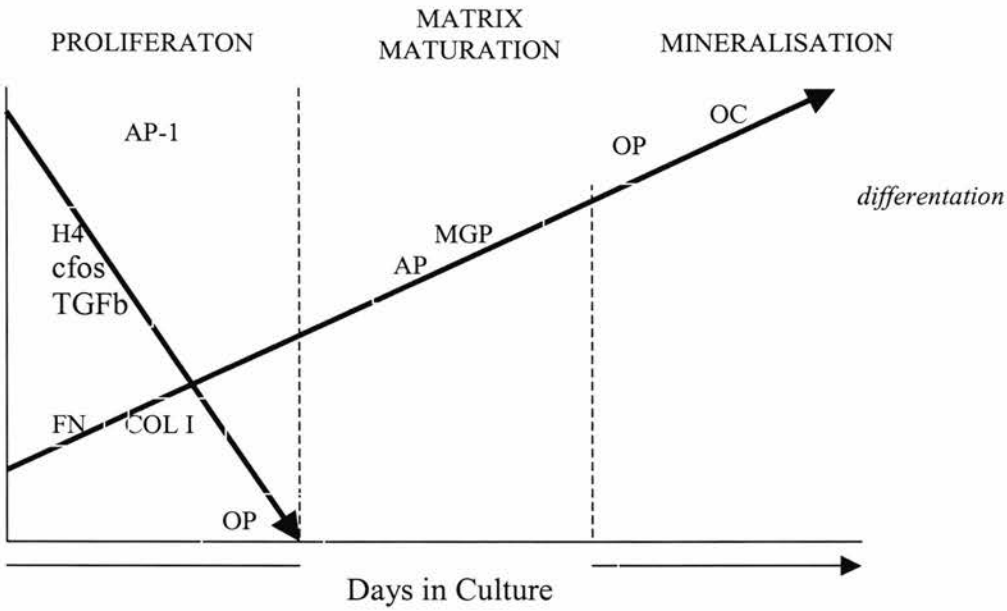


Figure 5.1 A diagram representing changes in expression of proliferation and maturation associated genes during the development of the osteoblast phenotype. (adapted from Lian and Stein 1996)

Developmental gene expression is part dependent upon position within the osteoblast lineage. There are two principle transition points, which are key for temporal expression of genes that support the progression of differentiation. The first point, completion of the proliferation period when genes for cell cycle and cell growth control are down regulated, and expression of genes encoding proteins for extracellular matrix maturation and organisation are initiated (Lian and Stein 1996). The absence during the proliferation period of gene expression observed in post-proliferative mature osteoblasts is called ''genotype suppression '' (Lian et al 1991).

The second transition point is at the onset of the expression of genes required for extracellular matrix mineralisation. Both transition points have been experimentally established and functionally defined as restriction points during osteoblast differentiation to which developmental expression of genes can proceed but cannot pass, without additional cellular signalling (Owen et al 1990).

5.2: Aims

1) To establish if differences in osteoblast characteristics such as cell proliferation and differentiation exist between cells isolated from the fast and slow growing strains.

2) To compare expression of genes involved in matrix synthesis (collagen type I, osteonectin, osteopontin and osteocalcin), transcription control (Runx2), growth (IGF-I and IGF-I receptor) and weight bearing (eNOS, glutamate receptor and serotonin receptor) between the two populations of osteoblasts. Such information may to help explain the differences in osteonal infilling rates noted previously, (chapter 3).

5.3: Methods

5.3.1: Bird housing and number for culture

Four birds kept in floor pens from both the fast (n=4) and the slow (n=4) strain, were culled at 21 days for bone extraction to establish osteoblast cultures as described in chapter 2 (section 2.2.1).

5.3.2: *In-situ* RNA analysis

The expression of genes which were differentially expressed in cell culture experiments (BSP, OPN, serotonin receptor and Runx2) were also examined *in situ* by LCM and RT-PCR. All RNA collected during LCM from the fast and slow growing strains were analysed for quality using an agilent bioanalyser, carried out by the ARK-Genomics (www.ark-genomics.org).

5.4: Results

5.4.1: *In Vitro* osteoblast analysis

The initial osteoblasts extracted from cortical bone through a series of digests were split into two populations (chapter 2, section 2.2.1). Those from digest 1 and 2 were combined to give population 1/2 and those from digest 3 and 4 were combined to give population 3/4. There was a visibly distinct difference between the two populations, with population 1/2 showing a mixed cell population with a high incidence of foam like cells whereas population 3/4 were more homogenous and representative of the osteoblast phenotype (Fig 5.2). Unfortunately one of the slow growing strain osteoblast cultures was lost due to contamination, leaving 3 cultures from the slow growing strain and 4 from the fast growing strain for analysis.

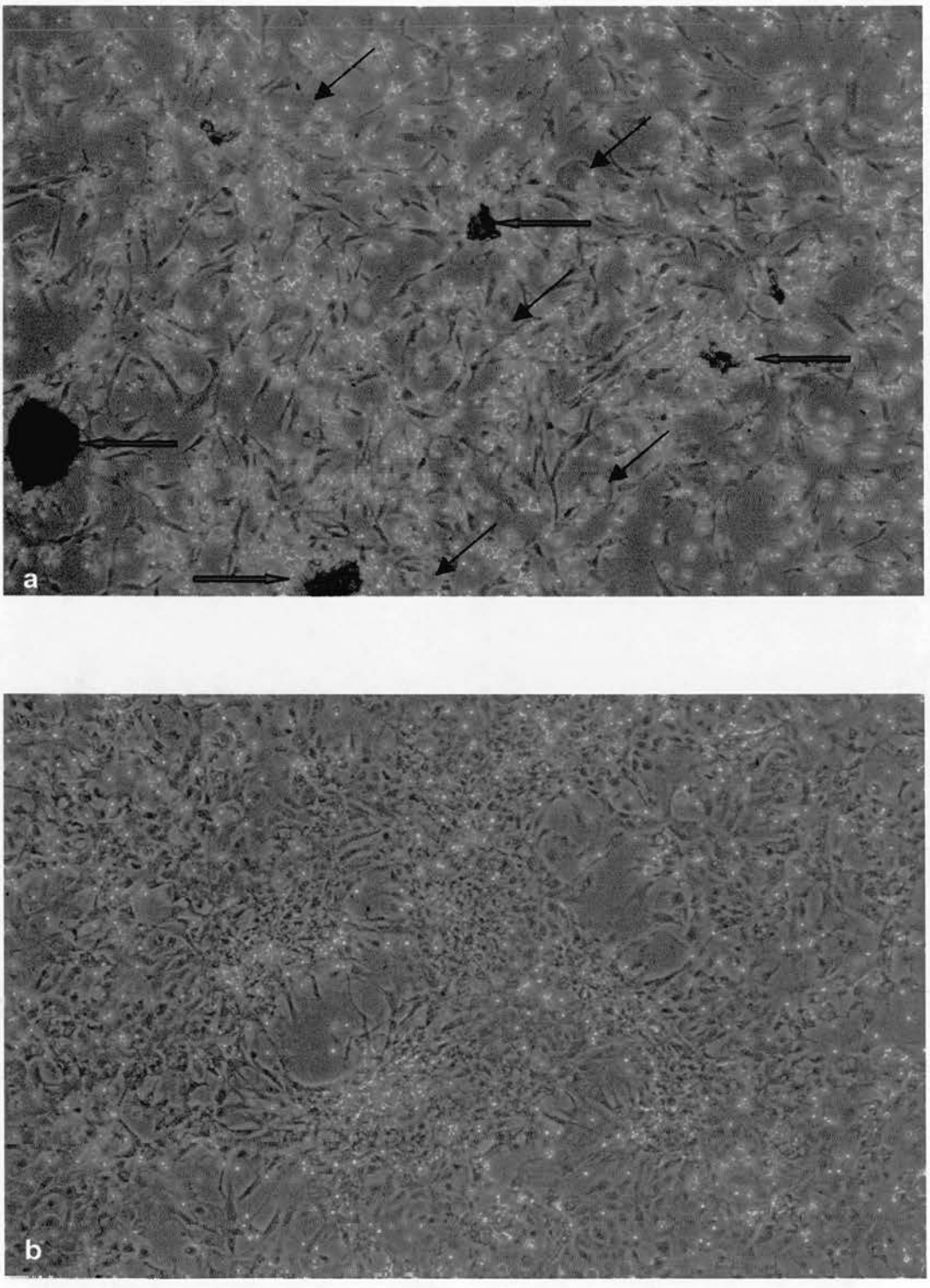
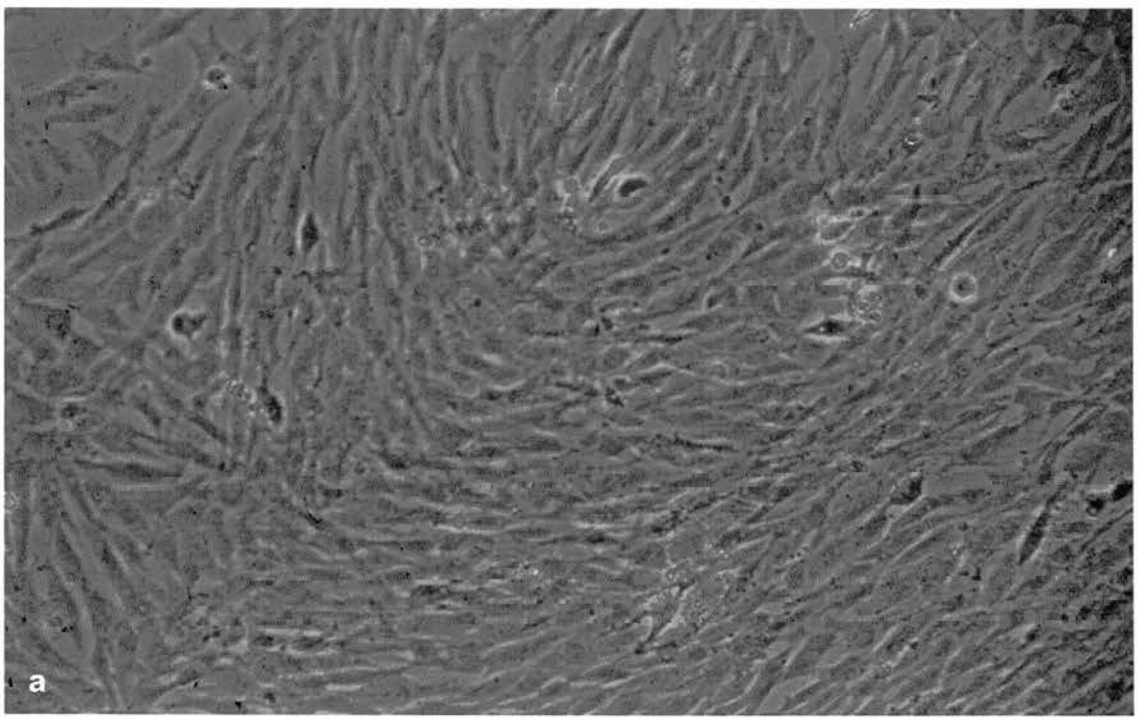


Figure 5.2 Digest populations 1/2 (a) and 3/4 (b) from initial primary culture after 4 days in culture. Red arrows indicate bone particle, black arrows indicate foam cells. Original magnification x100.

Population 3/4 also showed a visible increase in the proliferation rate of those extracted from the slow growing chickens compared to those from the fast growing chickens (Fig 5.3). As the population appeared to be more representative of the osteoblast phenotype, cells from this population of the samples were used for the *in vitro* studies. Cells in population 1/2 were discarded.



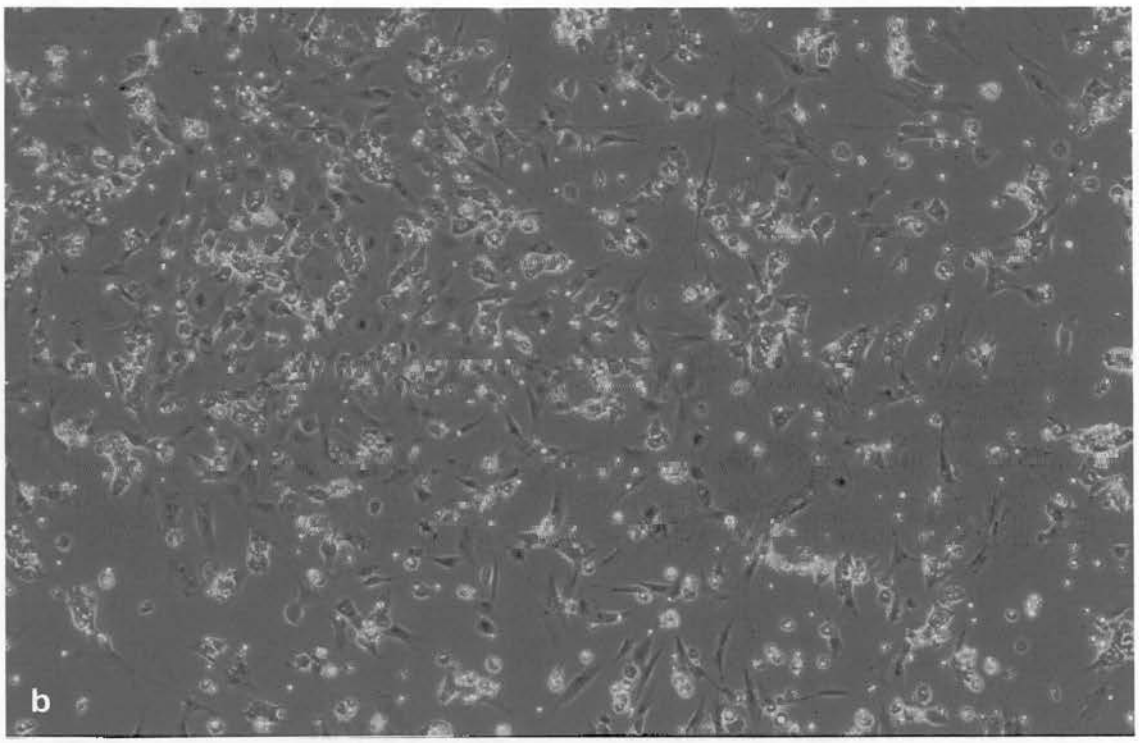


Figure 5.3 Visible difference in the number of osteoblasts extracted (population 3/4) from slow (a) and fast (b) growing strains after 4 days in culture. The cells were plated at the same density. Original magnification x100.

5.4.2: Osteoblast proliferation

Osteoblast proliferation was determined by tritiated-thymidine uptake. At the pre-confluency stage (T1,3days), cell proliferation (dpm) was higher in the slow growing birds ($F = 7439 \pm 287$; $S = 11732 \pm 371$; $P<0.001$), but this pattern was reversed at confluency (T2,7days) ($F = 10491 \pm 1290$; $S = 1979 \pm 322$ $P<0.001$) and post confluency (T3,11days) ($F = 4564 \pm 565$; $S = 1702 \pm 57$; $P<0.001$). These latter observations at T2 and T3 are possibly a consequence of the earlier impairment of proliferation by contact inhibition in the slow growing strain (Fig 5.4).

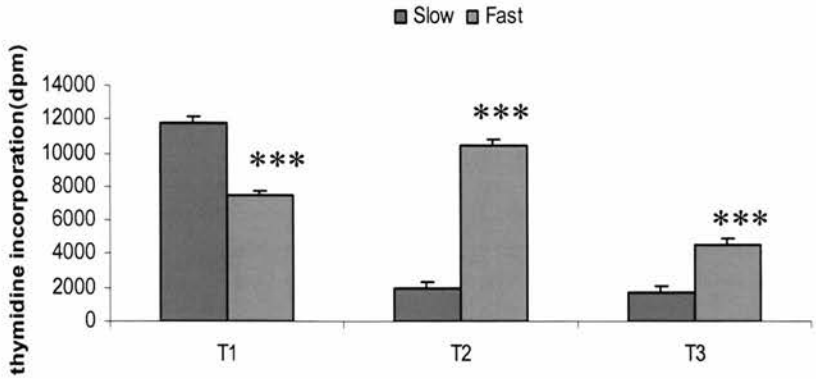


Figure 5.4 Osteoblast proliferation rate in pre-confluent (T1,3days), confluent (T2,7days), and post-confluent (T3,11days) cultures. Data are shown as mean ± SEM, ***P<0.001.

5.4.3: Osteoblast differentiation

Determination of differentiation was assessed by ALP activity. ALP activity (pNPP hydrol/30 min/mg protein) was only detected at the post-confluency stage and was significantly higher in the fast growing strain (F = 1188 ± 197; S = 216 ± 185; P<0.001) (Fig 5.5).

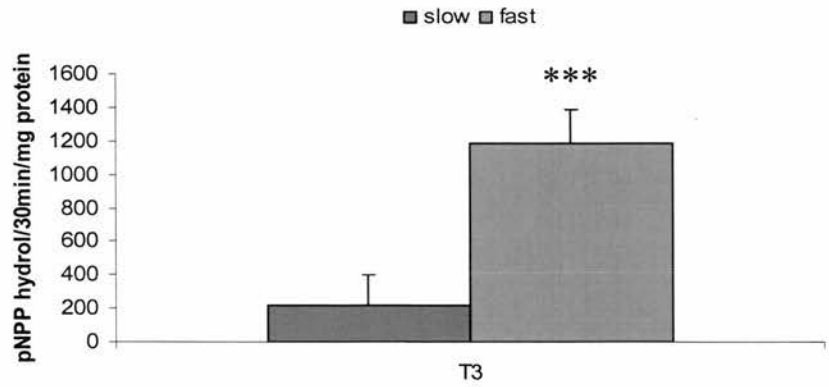


Figure 5.5 Osteoblast ALP activity in post-confluent (T3,11days) cultures. Data are shown as mean ± SEM, ***P<0.001.

5.4.4: Gene Expression studies

Osteoblastic gene expression was determined by semi-quantitative RT-PCR and quantified by densitometry (cnt/mm^2). A higher level of osteopontin (F = 382 ± 79; S = 1205 ± 81, P< 0.01) (Fig 5.6), and BSP (F = 33 ± 24; S = 262 ± 76, P<0.05) (Fig 5.7) expression was observed in osteoblasts derived from slow growing birds.

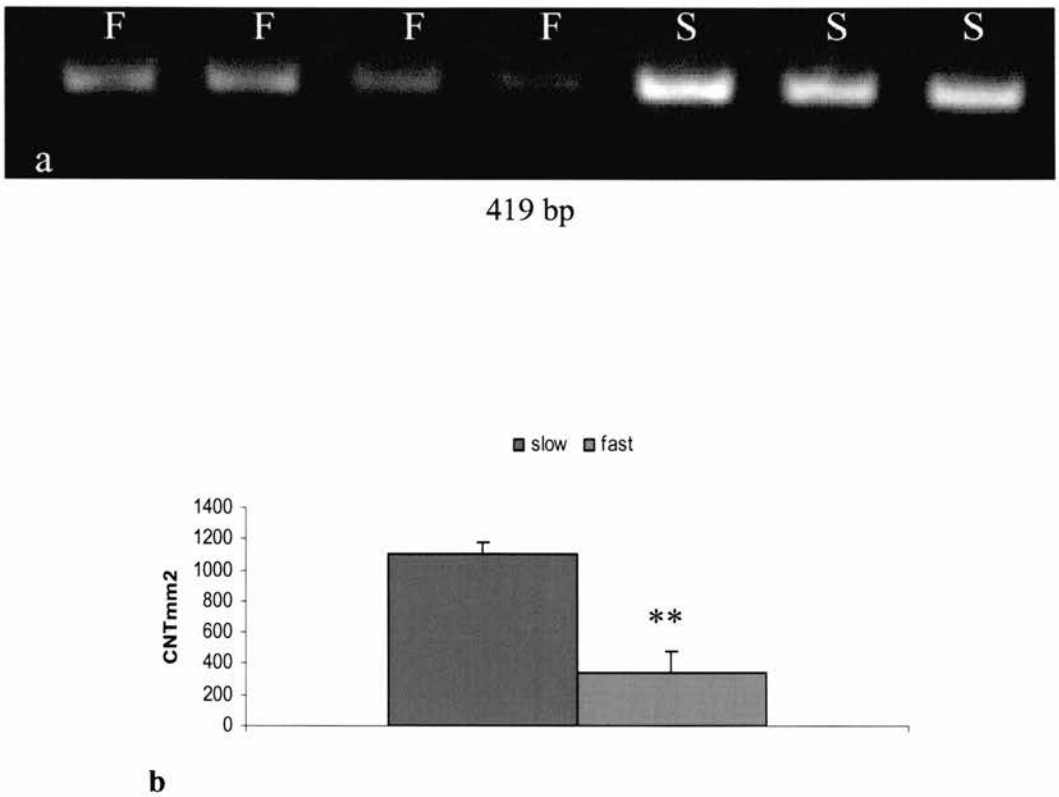


Figure 5.6 a) Expression of OPN in cultured osteoblasts from 4 fast (F) and 3 slow (S) growing birds by RT-PCR. b) Quantification of expression by densitometry at T3,11days. Data is shown as mean ± SEM, ** P<0.01

480 bp

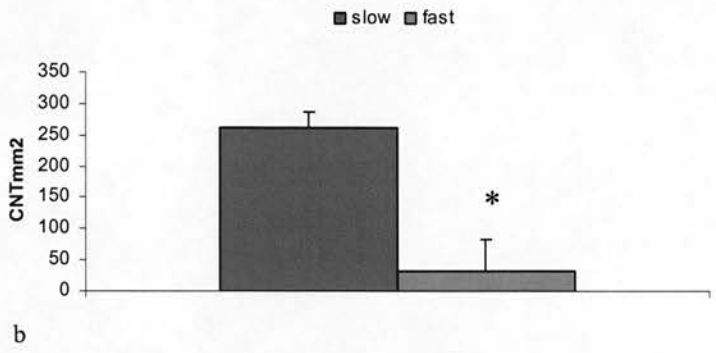
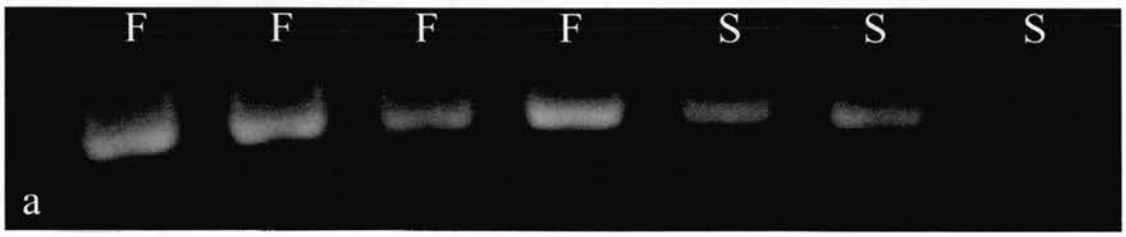
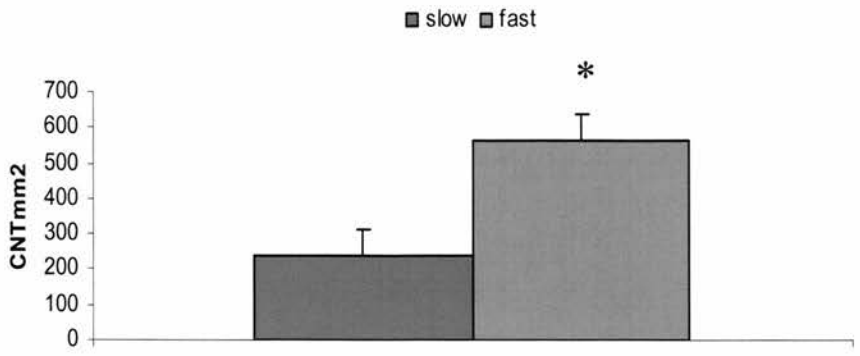


Figure 5.7 a) Expression of BSP in cultured osteoblasts from 4 fast (F) and 3 slow (S) growing birds by RT-PCR. b) Quantification of expression by densitometry at T3,11day. Data is shown as mean ± SEM, * $P<0.05$.

The serotonin receptor, considered to have a role in mechano-regulation, was more highly expressed in osteoblasts from fast growing chickens (cnt/mm^2) (F = 565 ± 74; S = 241 ± 74, $P<0.05$) (Fig 5.8) as was Runx2 (F = 312 ± 9; S = 72 ± 35; $P<0.001$) (Fig 5.9)

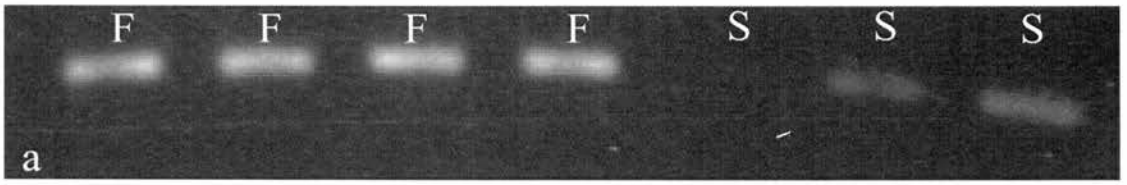


420 bp

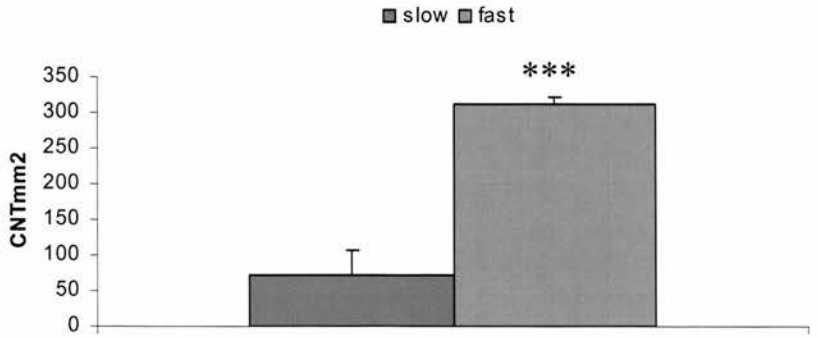


b

Figure 5.8 a) Expression of bone serotonin receptor in cultured osteoblasts from 4 fast (F) and 3 slow (S) growing birds by RT-PCR. b) Quantification of expression by densitometry at T3,11day. Data is shown as mean ± SEM, * P<0.05.



364 bp



b

Figure 5.9 a) Expression of Runx2 in cultured osteoblasts from 4 fast (F) and 3 slow (S) growing birds by RT-PCR. b) Quantification of expression by densitometry at T3,11day. Data is shown as mean ± SEM, ***P<0.001.

The house keeping gene GAPDH was used as a control. As expected it was equally expressed in all samples from the fast and slow growing strains (cnt/mm^2) (F = 107.8 ± 35; S = 102.1 ± 26) (Fig 5.10).

300 bp

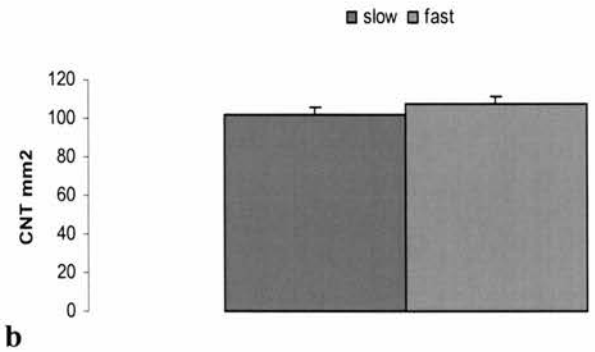


Figure 5.10 a) Expression of GAPDH in cultured osteoblasts from 4 fast (F) and 3 slow (S) growing birds by RT-PCR. b) Quantification of expression by densitometry at T3,11day. Data is shown as mean ± SEM.

The expression of Collagen type I (cnt/mm^2) (F = 186 ± 32; S = 250 ± 46), (Fig 5.11) osteonectin (F = 1400 ± 109; S = 1300 ± 67) (Fig 5.12), osteocalcin (F = 118.3 ± 70; S = 187.4 ± 16) (Fig 5.13) and ER α (F = 946.5 ± 85; S = 921.7 ± 214) (Fig 5.14) was not different between the strains. Unfortunately with continued trial of temperature and cycle number changes, no results could be obtained with primers to

eNOS, IGF-1, Glutamate receptor (NMDA), and IGF1-receptor. Due to time limitations these studies were not taken further.

351 bp

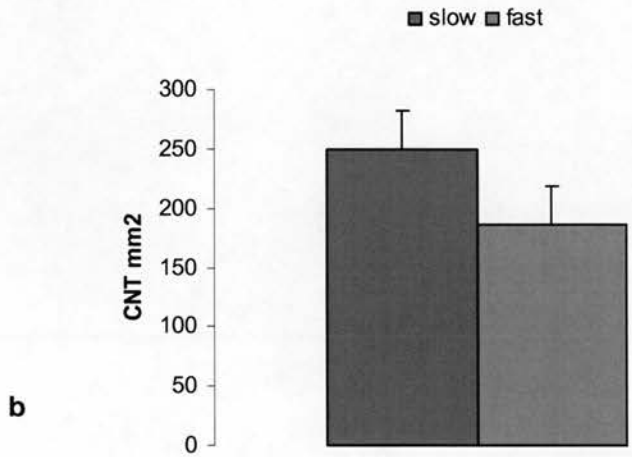
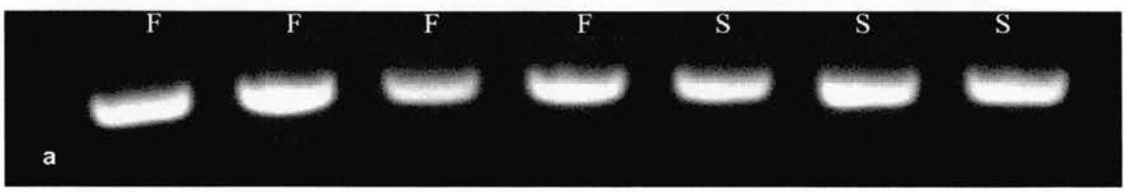


Figure 5.11 a) Expression of COL1A1 in cultured osteoblasts from 4 fast (F) and 3 slow (S) growing birds by RT-PCR. b) Quantification of expression by densitometry at T3,11day. Data is shown as mean ± SEM.



439 bp

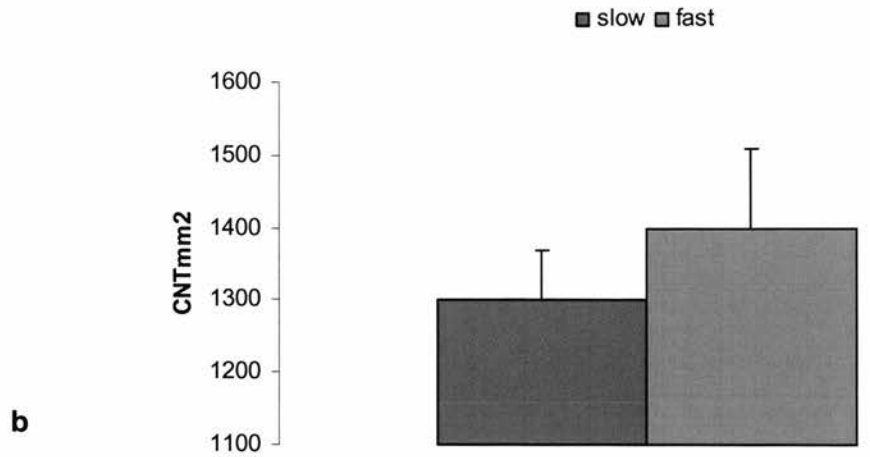
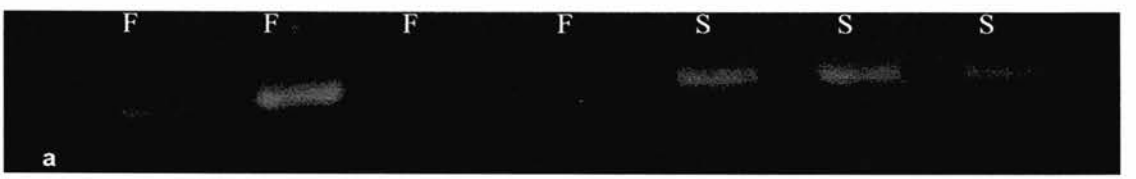


Figure 5.12 a) Expression of osteonectin in cultured osteoblasts from 4 fast (F) and 3 slow (S) growing birds by RT-PCR. b) Quantification of expression by densitometry at T3,11day. Data is shown as mean ± SEM.



318 bp

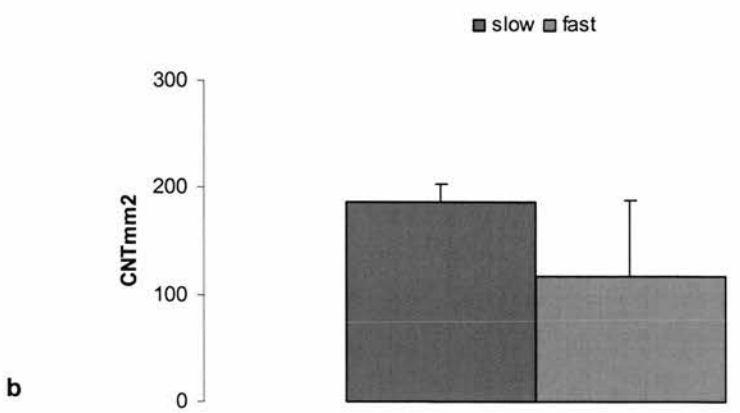


Figure 5.13 a) Expression of osteocalcin in cultured osteoblasts from 4 fast (F) and 3 slow (S) growing birds by RT-PCR. b) Quantification of expression by densitometry at T3,11day. Data is shown as mean ± SEM.

320 bp

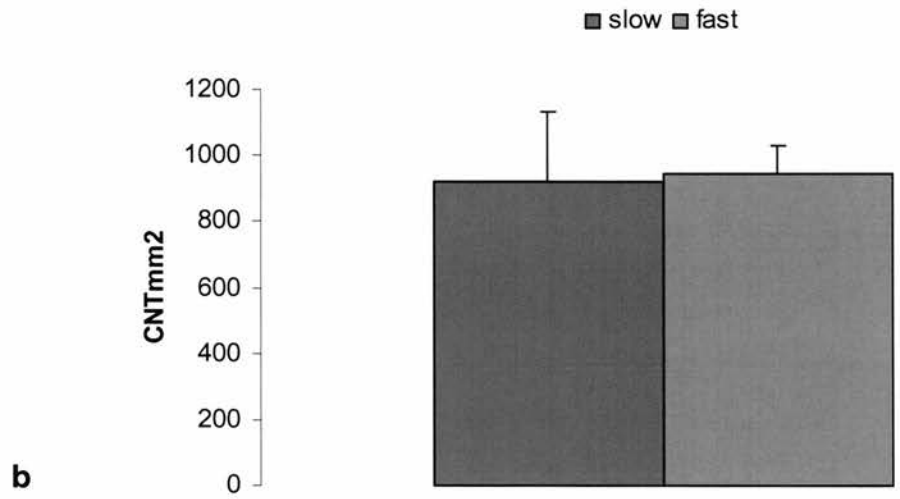


Figure 5.14 a) Expression of ERα in cultured osteoblasts from 4 fast (F) and 3 slow (S) growing birds by RT-PCR. b) Quantification of expression by densitometry at T3,11day. Data is shown as mean ± SEM.

5.4.5: Laser Capture Microscopy

5.4.5.1: Validation of LCM protocol

RNA extracted from chondrocytes of the hypertrophic (Fig 5.15) and proliferating (Fig 5.16) regions of a chicken growth plate by LCM, were amplified by RT-PCR using primers against collagen type II and X (chapter 2, section 2.2.12). Collagen type II expression was seen in both maturational zones, where as collagen type X

expression was present only in the cells of the hypertrophic zone (Fig 5.17). This data was consistent with the known expression of collagens II and X in the growth plate (Farquharson et al 2001). This confirms that the LCM methodology was viable in detecting differences in gene expression.

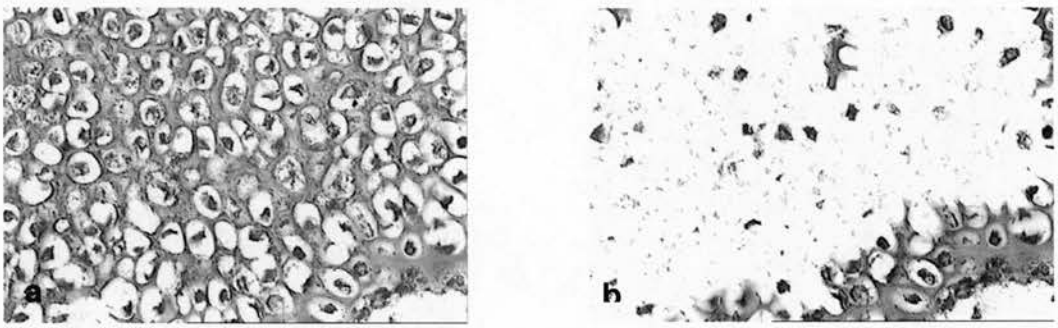


Figure 5.15 Cells at the hypertrophic region of the growth plate before laser capture (a) and the same site after laser capture (b).

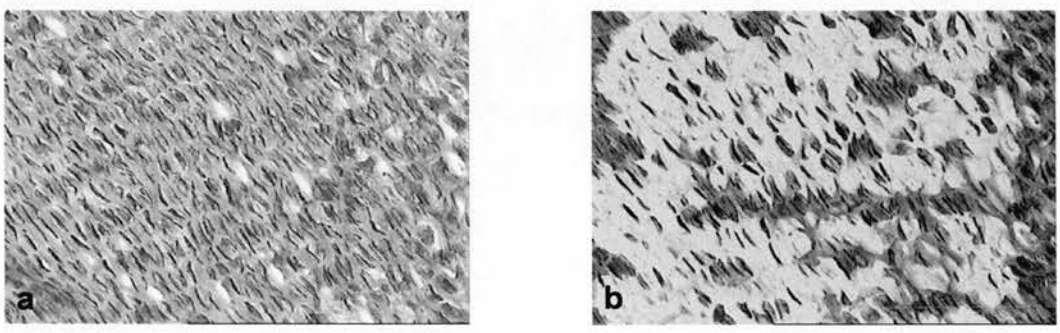


Figure 5.16 Cells at the proliferating region of the growth plate before laser capture (a) and the same site after laser capture (b).

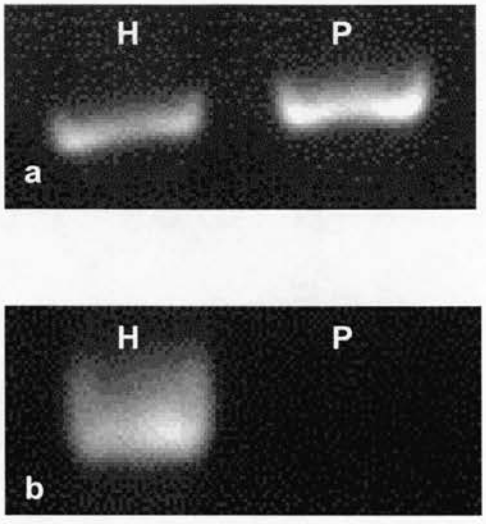


Figure 5.17 (a) collagen type II and (b) collagen type X expression in hypertrophic (H) and proliferating (P) chondrocytes of the growth plate.

5.4.5.2: Laser Capture Microscopy

It was my intention to extend the *in vitro* gene expression studies described in this chapter to the *in vivo* situation. This was to be done by isolating RNA from osteonal osteoblasts from the fast and slow growing strains and looking at the expression of the genes that had altered expression in the *in vitro* studies *i.e*; OPN, BSP, serotonin receptor and Runx2. Although cells were successfully targeted in both fast growing chickens (Fig 5.18) and slow growing chickens (Fig 5.19) and RNA extracted, a result could only be obtained when the samples were run with 18S primers (Fig 5.20).

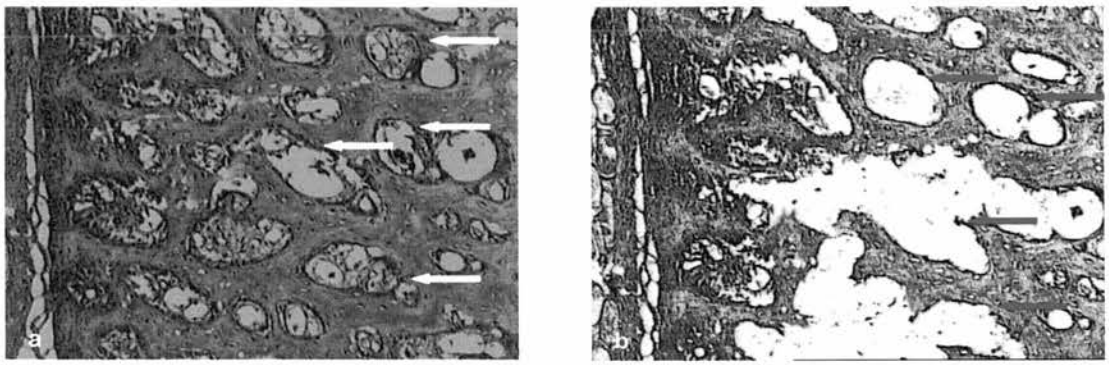


Figure 5.18 Osteons at the periosteal edge of a fast growing chicken cortical bone before osteoblast cell extraction by laser capture (a) and after laser capture of cells (b). White arrows indicate osteoblasts within the primary osteons, and blue arrows indicate where cells have been removed. All arrows point towards the periosteal edge.

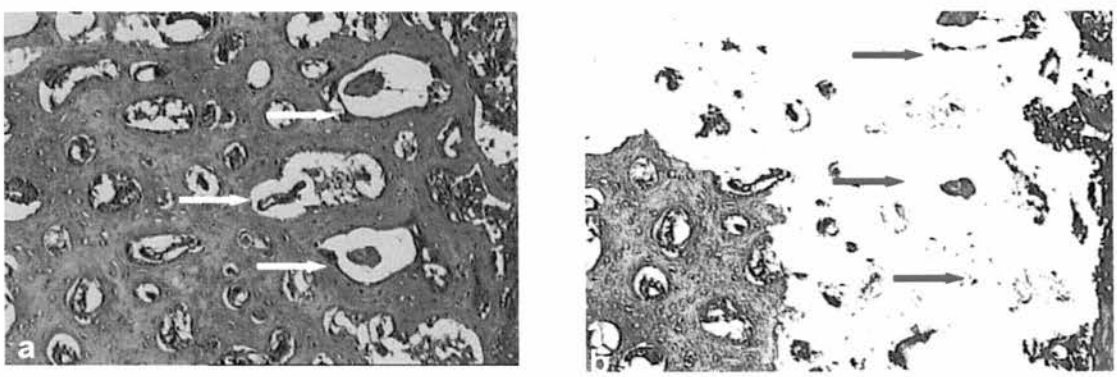


Figure 5.19 Osteons at the periosteal edge of a slow growing chicken cortical bone before osteoblast cell extraction by laser capture (a) and after laser capture of cells (b). White arrows indicate osteoblasts within the primary osteons, and blue arrows indicate where cells have been removed. All arrows point towards the periosteal edge.

All samples were originally tested using the same RNA concentration per reaction as was carried out in the *in vitro* studies chapter 2 (section 2.2.8). As this yielded no results the concentration level of the RNA per reaction was increased to that stated in chapter 2 (section 2.2.10), however this still produced no product. The number of cycles per reaction was then increased but unfortunately there was still no product

produced by RT-PCR analysis. The right and left bone samples from each individual bird were then combined to give one sample per bird, and the RNA concentrated. Again no product was produced on analysis. All the fast growing samples were then combined together to give a single RNA sample, as were the samples from the slow growing chickens combined into one single sample. Once again there was no product produced. All samples were tested at each stage by Agilent (appendice 1), which showed the samples to be degrading. From capture with the LCM to these various analysis stages took several months and time prohibited starting this again. Therefore no results were obtained by this method for the analysis of *in-situ* gene expression.

Figure 5.20 Expression of 18S from LCM collected RNA from 4 fast (F) and 4 slow (S) growing birds of the right tibiotarsi by RT-PCR.

5.4.6: Immunoblotting

Protein extracted from cultured osteoblasts tested against collagen type I showed a single band in all samples of fast and slow growing chickens with no difference between strains (Fig 5.21). Several other antibodies were tested as listed in chapter 2 (section 2.2.7), but no bands were detected.

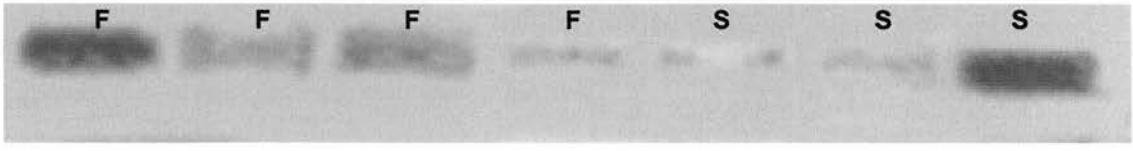


Figure 5.21 Collagen type I protein expression from cultured osteoblasts from fast and slow growing chickens.

Protein extracted from cultured osteoblasts reacted with antibodies to BSP. There was a significant difference in expression levels by the cells from the fast and slow growing strains of chicken. The slow growing chickens had a higher expression of BSP than that of the fast growing strain when quantified by densitometry (cnt/mm^2) ($F = 3453 \pm 48.3$, $S = 4274 \pm 40.6$; $P < 0.01$) (Fig 5.22).

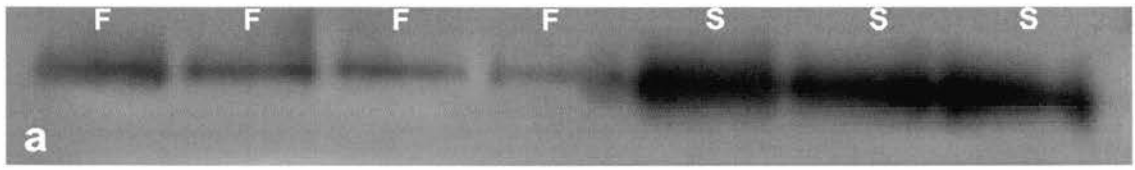


Figure 5.22 BSP protein expression from cultured osteoblasts from fast and slow growing chickens.

5.5: Discussion

Osteoblasts isolated from slow growing chickens proliferated in culture significantly faster at pre-confluency than osteoblasts from fast growing chickens. These results are consistent with the *in vivo* data of chapter 4 where a higher proliferation rate was noted within periosteal cells of the slow growing chickens. During osteoblast proliferation an increase in the expression of growth factors such as IGF-1(Birnbaum and Wiren 1994) and collagen type I (Stein and Lian 1995) have been reported. Further, it has been shown that the systemic levels of IGF-I in slow growing chickens (as used in this study) are actually higher than that of the fast growing chickens (Goddard et al 1988). It has been reported that bone growth rate can be expressed as the product of cell production rate (Kirkwood et al 1989). This is supported by the results of this study where the proliferation rate was higher in the osteoblasts from the slow growing chickens which produce a faster rate of bone formation within the

newly formed primary osteon at the periosteal surface (chapter 4). This increased proliferation may be a result of the higher levels of IGF-I (Goddard et al 1988) in the slow growing chicken. IGF-I is a known mitogen for several cell types, including osteoblasts (Canalis 1980).

ALP activity post confluency (11 days) was higher in osteoblasts from the fast growing chickens than those from the slow growing chickens. This would be consistent with osteoblasts from the fast growing strain exiting the cell cycle faster and entering the differentiation pathway. This would explain the lower proliferation rates and higher ALP activity in the osteoblasts from the fast growing chickens. The changes in cell shape promoted by the multi-layering of cells in culture may provide signals for osteoblast differentiation. The effect of crowding on cell proliferation, a phenomenon mediated by cell shape and contact has been discussed by Folkman and Moscona (1978). It has also been reported that osteocalcin expression by osteoblasts mirrored that of ALP activity in osteoblast cultures (Gerstenfeld et al 1987, Aronow et al 1990). This was not found in the present study where osteocalcin expression was similar in osteoblasts from slow and fast growing chickens.

In culture, isolated osteoblasts have, not surprisingly, been shown to synthesize several proteins and enzymes known to be localised to bone (Aubin et al 1982, Ashton et al 1985, Gerstenfeld et al 1987). Cellular differentiation of cultured cells is suggested by the apparent increase in the expression of protein phenotypic bone markers. The expression of OPN, osteonectin, osteocalcin and BSP is dependent on the stage of osteoblast differentiation. Osteonectin is expressed by cells localised to immature osteoid and close to the bone mineralisation front (Jundt et al 1987, Romanowski et al 1990). In addition to a possible role in matrix mineralisation it has also been reported to be involved in the regulation of cell proliferation (Mundlos et al

1992, Nakase et al 1994). In contrast, osteocalcin synthesised by osteoblasts is limited to highly mineralised regions of the bone matrix (Mark et al 1988). OPN has been localised to cells in mineralising tissues suggesting it is involved in the mineralisation process (Mark et al 1988, Boskey 1992). Its localisation in bone resembles published data on the distribution of BSP mRNA (Chen et al 1992).

Cells cultured from the leading edge of chicken embryo tibiotarsi as done in this present study, have been shown to be committed to the osteogenic lineage (Nurminskaya et al 2003). The cells express a number of osteoblastic markers such as transcription factor Runx2 and the matrix proteins collagen type I, osteonectin and OPN (Quarles et al 1992, Ducy et al 1997). These cells did not express other characteristics indicative of overtly differentiated osteoblasts such as BSP and osteocalcin (Raouf and Seth 2000). Such cells were classed as pre-osteoblasts (Nurminskaya et al 2003). This implies that the osteoblasts from the fast birds are further down the osteoblast differentiated pathway. This is backed up by the immunoblotting results, which also show a higher level of BSP in the slow growing chickens compared to that of the fast growing chickens. This is in contrast to the ALP data which was greater in the osteoblasts from the fast growing chickens and therefore it may be too simplistic to think that the high ALP activity in cells of the fast strain is a consequence of cell cycle exit and the initiation of differentiation. Alternatively, the osteoblasts of the slow growing strain proliferate rapidly and enter the differentiation cascade upon reaching confluency at an earlier stage than the osteoblasts from the fast growing chickens. This would explain the complete reversal of the proliferation rates of the osteoblasts from the fast and slow chickens at pre-confluency (3 days) and post-confluency (11 days). It has been reported that as osteoblasts cease proliferation and initiate the differentiation cascade, there is a large

increase in OPN expression (Zohar et al 1997). This is in agreement with the suggestion that the osteoblasts from the fast growing chickens are slower in initiating the differentiation process.

In contrast, the fast growing chickens had a higher expression level of Runx2 compared to that of cells from the slow growing chickens. Several laboratories have shown that Runx2 is highly expressed in osteoblast cells (Ducy et al 1997, Komori et al 1997) and regulates the expression of various osteoblast genes (Banerjee et al 1997, Ducy et al 1997, Tsuji et al 1998). Targeted disruption of Runx2 in mice has shown that it is an essential transcription factor in osteoblast differentiation (Liu et al 2001). Other studies have shown that late stage osteoblast maturation is inhibited by over-expression of Runx2 and this is associated with an accumulation of OPN and with decreased expression of osteocalcin and ALP (Liu et al 2001). An accumulation of less mature osteoblasts in mice over-expressing Runx2 appeared to be caused by maturational blockage of the osteoblasts but also by acceleration of osteoblast differentiation at an early stage of cell development due to the increased number of osteoblasts at neonatal stage (Liu et al 2001). This could also explain my results where in chapter 4 I showed a higher number of cells within the periosteum of the fast growing strain available for osteonal incorporation but with a lowered proliferation rate. Although ALP activity can be detected at an early stage of osteoblast differentiation differences in ALP expression were not noted at early neonatal stages but were more evident as development progressed (Stein et al 1990). This evidence suggests that the over-expression of Runx2 blocks osteoblast maturation at a certain stage or developmental window in the osteoblast lineage (Liu et al 2001). Over-expression of Runx2 at a late stage of osteoblast differentiation caused a decrease in the bone formation rate and a decrease in the expression of the

major genes encoding bone matrix proteins (Ducy et al 1999). This data strengthens the hypothesis (proposed in chapter 4) that osteoblasts at the periosteal edge of the cortical bone in the fast growing chickens do not reach late stage differentiation, taking the hypothesis further by now saying this is due to the high levels of Runx2. Therefore they do not lay down the required amount of bone matrix necessary to infill the large primary osteons. This hypothesis could also explain the increased osteocyte density within the circumferential lamellae, noted in chapter 4. This could be either by maturational blockage of the osteoblasts and/or by acceleration of osteoblast differentiation at an early stage of cell development.

The characterisation of serotonin receptors in chicken bone has received little attention. One published paper (Westbroek et al 2001) investigated the expression of serotonin receptors in cultures of both osteoblasts and osteocytes. The receptor 5-HT $_{2B}$ has been noted as a vasodilator on endothelial cells (Ullmer et al 1995, Glusa and Roos 1996). In relation to this study the osteoblasts are within the primary osteons together with incorporated blood vessels that are modulated by blood flow and blood pressure. Mechanical loading causes the flow of interstitial fluid through the canalicular system as well as that of the primary osteons. The pulsatile movement of interstitial fluid is sensed by the osteocytes and translated into signalling molecules such as NO (Klein-Nuland et al 1995a,b) with these signalling molecules regulating the effectors of bone adaptation (Burger and Klein-Nuland 1999). With relation to osteoblasts it was found that they contain 5-HT $_{2B}$ receptor (the specific sub-type examined in this study), with the serotonin inhibiting fluid flow induced NO (Westbroek et al 2001). Unfortunately in this study the determination of expression levels of eNOS were unsuccessful and no product was obtained using previously examined primers Fujii et al (1998). Therefore, the significance of the difference

shown between the two strains in 5-HT $_{2B}$ receptor expression remains unclear and further studies are warranted.

The LCM methodology was obviously limiting in the case of the tibiotarsi. As positive results were obtained from the growth plate sections it is likely that this was due to a greater cell number available for collection. In order to try and maximum cells I captured four areas from one section with two sections used per bone. However as the results showed this was still not enough osteoblasts to give a sufficient RNA for analysis in the case for the tibiotarsi section. For further work additional areas from more sections would be required to obtain sufficient RNA for analysis. A further improvement to the procedure would be the inclusion of a two step RT-PCR instead of a one step process as used in this study. This would enable further amplification of RNA collected to gain a greater product.

5.6: Conclusion

In conclusion, cultured osteoblasts showed distinct differences in their rate of proliferation and differentiation and the expression levels of genes and protein between the two strains of chicken. The ALP data suggests that osteoblasts from the fast growing chickens differentiate towards the terminally differentiated phenotype faster than the cells from the slow growing chickens. However, the results from the OPN, BSP and Runx2 gene expression studies indicate that the osteoblasts from the fast growing chickens may proceed through the osteoblast differentiation cascade slower than those from the slow growing chickens. Both scenarios may result in abnormally low levels of matrix production by the fast growing osteoblasts and this may account for the slower infilling rate within the primary osteons of the fast growing strain of chicken. Unfortunately, the hydroxyproline assay used in this study

did not appear to be sensitive enough to measure collagen production by both sources of cells and therefore the direct measurement of collagen production was not possible.

Chapter 6: General Discussion

6.1: Porosity

Cortical porosity is not unique to the immature skeleton of the fast growing birds studied in this thesis. It also plays a significant role in humans. In normal healthy adults the amount of cortical bone that is resorbed and formed is in balance. With onset of age and /or disease there is evidence that bone loss occurs often resulting in fractures of the proximal femur or spine. Many studies indicate that this is due to reduced cancellous bone formation (Parfitt et al 1983, Erikson 1986). However only a few studies have examined changes in cortical bone remodelling with increasing age or estrogen loss (Thompson 1980, Bell et al 1999a). However recent studies by Bell and colleagues (2000) confirmed that the increased cortical porosity observed in the femoral neck of fracture cases was due to increasing Haversian canal size and canal number originally observed by Atkinson 1964, Thompson 1980. The increased porosity was mainly due to actively remodelling canals in fracture cases. Both cortical porosity and Haversian canal size have been shown to have implications for bone strength and stiffness (Barth et al 1992, Currey et al 1996, Yeni et al 1998). Although remodelling was not the cause of the porosity in this study, the increased porosity in humans is associated with increased fractures. A similar scenario may occur in the fast growing birds of this study. It has been noted in this study that there are fast and slow growing areas of the cortical bone within the tibiotarsi. These areas appeared to be dependent on whether the bone was under tension or compression. A study within humans has also indicated regional differences in cortical porosity of fractured femoral necks (Bell et al 1999b). Cortical bone that was in tension had a higher porosity than that which was in compression. In cases of human femoral fracture a greater increase in cortical porosity has been shown which was due to a higher proportion of canals being remodelled in the tension area (Bell et al 1999b). It

was also the tension side of the cortical bone within the fast growing chickens of this study which was expanding rapidly to adapt to the birds weight. The compressed cortex of mule deer calcaneus has also been show to have a lower rate of remodelling than that of the tension side of the cortex (Skedros et al 1994). However these findings are in contrast to tension/compression studies in sheep, where sheep tibia showed no cortical differences despite the strain mode (Lanyon and Bourn 1979). Also, horse radii showed no difference between the tension and compression cortices (Riggs et al 1993a,b). These differences may represent alternative structural/material adaptations. However, sheep tibia and horse radii do not have a plantar ligament present, as is the case for the mule deer calcaneus, and this may alter the loading conditions present (Skedros et al 1994). It is also unclear whether direct comparisons between short cantilevered bones and long limb bones are appropriate since different regions of the same bone may be sensitive to different strain magnitudes (Skedros et al 1994).

It has also been recently shown that remodelling osteons are clustered together in the femoral head (Jordan et al 2000). The presence of composite systems was significantly dependent on the regional distribution of clusters of remodelling osteons. Osteoclastic resorption in closely adjacent osteons could lead to merging of Haversian canals and therefore these composite systems may represent canals that, in three dimensions, are moving closer together (Jordan et al 2000).

6.2: Osteocyte Influences

Osteocytes differentiate from osteoblasts that have become buried in osteoid during bone formation. It has been theorised that an inhibitory signal travelling through the canalicular processes from the osteocytes to the osteoblasts initiates recruitment of

selected osteoblasts into the osteocyte lineage (Marotti et al 1995). This theory was extended to explain the nonlinear refilling rate in secondary osteons as a result of a more general inhibitory signal acting on the entire population of osteoblasts at the refilling surface (Martin 2000). These authors hypothesised that the strength of the inhibitory signal perceived by each osteoblast is proportional to the osteocytes in communication with it and inversely proportional to the distances involved (Martin 2000). This hypothesis was also found to be consistent with earlier studies that showed declining apposition rate during the course of bone formation in newly forming osteons (Manson and Waters 1965). If there is some critical level of the inhibitory signal at which osteoblast cease their function and either apoptose or differentiate into osteocytes or bone lining cells, the osteocyte population density should also determine the duration of filling and thickness of the completed osteonal wall (Metz et al 2003). The latter is important because it affects the distance that nutrients must travel from the Haversian canal to reach the outermost osteocytes after the osteon is complete. Therefore according to the hypothesis of an inhibitory signal recruiting osteoblasts to become osteocytes, the osteoid formation period would be less in high osteocyte density osteons, as infilling would occur quicker, and a high osteocyte density would halt infilling earlier. In this study the osteons of the fast growing chickens did not (chapter 4) have a higher osteocyte density than those of the slow growing chickens. On the basis of this hypothesis (Martin 2000) it could be suggested that a greater canal wall surface is available to support greater nutrient transport to the circumferential lamellae which had, in this case a higher osteocyte density. Statistically significant correlations between osteocyte density and osteon dimensions support the existence of an inhibitory signal and its effect on osteoblast function (Martin 2000). If such a mechanism has evolved that osteocytes will

sacrifice bone mass ensuring a larger canal (porosity) to protect survival of new bone, then osteocyte function is essential for other reasons than just mechanosensory and chemosensory functions. This is interesting when considering that the osteocytes may have some control over the osteoblasts, and the osteocytes are obviously under a greater loading strain in the fast growing chickens when compared to the slow growing chickens.

It was unfortunate that in this study no results were obtained for an estimate of NO expression by the detection of the synthase isoenzyme eNOS. This would have been an excellent indicator of the loading response to the increased body weight. Further investigations into the effects of NO within these birds would have been informative as NO isoenzymes have been shown to modulate bone resorption (Osbody et al 2000) and formation (Turner and Pavalko 1998). Given more time, other primers could have been designed and tested for the *in vitro* studies in addition to alternative antibodies to eNOS. Results to such studies would have been interesting when compared to those reported in other studies which indicted that bones of meat-type chickens, were resistant to loading (Pitsillides et al 1999). Further studies could also be carried out by weighting the slow growing birds, by fitting weighted rucksacks, to determine if the cortical bone would show similar characteristics to that of the fast growing birds. Investigations would also be extended to determine if the pattern of gene expression by osteoblasts was similar to that observed in the osteoblasts from the fast growing birds.

Given more time, it would have been desirable to study the incidence of apoptosis within osteoblasts of both the periosteum and the primary osteons. Published methods for the study of osteocyte apoptosis would be used (Noble et al 1997) rather than the molecular probes methods originally used in this study. This would

determine if the increased cell death was responsible for the lack of infilling within the primary osteons. Finally, to further the *in vitro* studies, it would have been beneficial to get the *in vivo* gene expression studies working using LCM. The method was validated on growth plates. Therefore it would not be unrealistic to consider that a small amount of technical adjustments would give a valid method for the bone sections.

6.3: Final Conclusions

Fast growing birds in this study have been selected since 1972 for greater body weight in the fastest possible time. The implications of this are that the fast growing birds have a weaker skeleton. In contrast, the slow growing birds have not been selected for any specific traits and allowed to develop as nature intended. The implications drawn from this study have shown that through selection for certain traits the fast growing birds now struggle to adapt its skeleton to meet the greater demands placed upon it. This was clearly shown in the reduced mechanical strengths and increased porosity from chapter 3. Perichondral development of the bone occurs in both strains of birds but is greater in the fast growing strain, however the infilling of the newly formed osteons from this process is lacking. Within the periosteum, proliferating cells are reduced in number in the fast growing strain (chapter 4) but this fact could be accounted for by osteoblasts laying down more bone periosteally than that of the slow growing birds. Previous studies (Williams et al 2004) have shown that the skeleton of fast growing birds can, through feed restriction, have similar morphology to that of the slow growing birds, implying that the problem is not genetic. However genetic studies (chapter 5) have shown distinct differences between the two strains of birds within some of the controlling genes of osteoblast

activity. This implies that due to the increased weight on the faster growing skeleton, some osteoblast genes and their activation/down regulation affect the growing immature skeleton. To achieve functional competence of the skeleton within the fast growing birds an immediate answer would be to feed restrict but in the current commercial situation this is not viable (chapter 5, 6.4), therefore further work on the Runx2, OP, BSP, and the serotonin receptor are needed to determine how their control is affected by mechanical loading.

6.4: Welfare Implications

There are a variety of health and reproductive problems in the modern meat-type chicken (Reddy 1996). Among the more common are skeletal disorders leading to lameness and other associated welfare and production problems. In a previous survey 90 % of the commercial meat-type birds were found to have a detectable abnormality in their gate (Kestin et al 1992) and skeletal problems are recognised as one of the four major factors affecting their performance (Day 1990). Short term periods of growth restriction were found to have no effect on bone quality at market age. Fundamental scientific investigations similar to those described in this thesis may help to provide answers to the bone fragility problems noted in modern meat-type burds. Identification of genes involved in osteonal formation during rapid growth may result in breeding strategies such as marker-assisted selection to improve bone quality. However in the meantime it is possible that the only way to improve bone quality in the modern meat-type chicken is to slow the growth rate by reversing genetic selection. This would result in economic implications for both the producer and the consumer and it is unclear if the majority of consumers would be willing to pay more in return for increased welfare.

Chapter 7: References

Ashton B A, Abdullah F, Cane J, Williamson M, Sykes B C, Couch M and Poser J W (1985). Characterisation of cells with high alkaline phosphatase activity derived from human bone and marrow: Preliminary assessment of there osteogenicity. Bone, 6:313-319.

Adamson E (1982). Collagen in Health and Disease, (Ed) Weiss J B and Jayson M V. (Churchill Livingstone, Edinburgh, UK).

Alberts B, Bray D, Lewis J, Raff M, Roberts K and Watson J (1994). Molecular biology of the cell, 3rd ed, Garland Publishing, London, UK.

Ali S Y, (1992). Bone biology and skeletal disorders in poultry. (Ed) Whitehead C C, (Carfax Publishing Company, Abingdon, UK).

Anderson H C, (1989). Biology of disease: mechanisms of mineral formation in bone. Laboratory Investigation, 60:190-197.

Armstrong D G and Hogg C O (1992). The expression of a putative insulin-like growth factor-I receptor gene I the liver of the developing chick. Journal of Molecular Endocrinology, 8:193-201.

Aronow M A, Gerstenfeld L C, Owen T A, Tassinari M, Stein G S and Lian J B (1990). Factors that promote progressive development of the osteoclast phenotype in cultured rat calvarial cells. Journal of Cellular Physiology, 143:213-221.

Aronson B D, Fisher A L, Blechman K, Caudy M and Gergen G P (1997). Groucho-dependent and independent repression activities of Runt domain proteins. Molecular Cell Biology, 17:5581-5587.

Ascenzi A and Bonucci E (1967). The tensile properties of single osteons. Anatomical record, 158:375-386.

Aubin J E, Heersche J N M, Merriless M J and Sodek J (1982). Isolation of bone cells clones with differences in growth hormone responses and extracellular matrix production. Journal of Cell Biology, 92:452-461.

Aubin J E (1998). Bone stem cells. Journal of Cell Biochemistry, 30:73-82.

Bab I A and Einhorn T A (1994). Polypeptide factors regulating osteogenesis and bone marrow repair. Journal of Cell Biochemistry, 55:358-365.

Balena R, Shis M and Parfitt A M (1992). Bone resorption and formation on the periosteal envelope on the ilium: a histomorphometric study in healthy women. Journal of bone and mineral research, 7:1475-1482.

Banerjee C, McCabe L R, Choi J Y, Hiebert S W, Stein J L, Stein G S and Lian J B (1997). Runt homology domain proteins in osteoblast differentiation: AML3/CBFA1is a major component of a bone-specific complex. Journal of Cell Biochemistry, 66:1-8.

Banks W J (1986). Applied veterinary histology. 2nd edition. (Williams and Wilkin, Baltimore, Hong Kong, London, Sydney)

Baron R, Neff L, Louvard D and Courtoy P J, (1985). Cell mediated extracellular acidification and bone resorption: evidence for a low pH in resorbing lacunae and localisation of a 100-kD lysosomal membrane protein at the osteoclast ruffled border. Journal of Cell Biology, 101:2210-2222.

Bar-Shavit Z, Teitelbaum S L, Reitsma P, Hall A, Pegg L e, Trial J and Kahn A J (1983). Induction of monocytic differentiation and bone resorption by 1α,25-dihydroxyvitamin D_3. Proceedings of the National Academy of Sciences USA, 80: 5907-5911.

Barth R W, Williams J L and Kaplan F S (1992). Osteon morphometry in females with femoral neck fractures. Clinical Orthop Rel Research, 283:178-186.

Baudet M-L, Sanders E J and Harvey S (2003). Retinal growth hormone in the chick embryo. Endocrinology, 144:5459-5468.

Bautista C M, Mohan S and Baylink D J (1990). Insulin-like growth factors I and II are present in the skeletal tissues of ten vertebrates. Metabolism, 39:96-100.

Bell K L, Loveridge N, Power J, Rushton N and Reeve J (1999a). Intracapsular hip fracture: increased cortical remodelling in the thinned and porous anterior region of the femoral neck. Osteoporosis international, 10:248-257.

Bell K L, Loveridge N, Power J, Garrahan N, Meggitt B F and Reeve J (1999b). Regional differences in cortical porosity in the fractured fermoral neck. Bone, 24:57-64.

Bell K L, Loveridge N, Jordan G R, Power J, Constant C R and Reeve J (2000). A novel mechanism for induction of increased cortical porosity in cases of intracapsular hip fracture. Bone, 27:297-304.

Beninati S, Senger D R, Cordella-Miele E, Mukherjee A B, Chackalaparampil I, Shanmugam V, Singh K and Mukherjee B B (1994). OPN: its transglutaminase-catalyised posttranscriptional modification and cross-linking to fibronectin. Journal of Biochemistry, 115:675-682.

Benjamin M and Hillen B (2003). Mechanical influences on cells, tissues and organs- mechanical morphogenesis. European Journal Of Morphology, 41:3-7.

Birnbaum R S, and Wiren D M (1994). Changes in insulin-like growth factor-binding protein expression and secretion during the proliferation, differentiation, and mineralization of primary cultures of rat osteoblasts. Endocrinology, 135:223-230.

Bloom W, Bloom M A, and McLean F C (1941). Calcification and ossification: Medullary bone changes in the reproductive cycle of female pigeons. Anatomical Record 81:443-453.

Bodine P V N, Henderson, R A, Green J, Aronow M, Owen T, Stein G S, Lian J B and Komm B S (1998). Estrogen receptor-α is developmentally regulated during osteoblast differentiation and contributes to selective estrogen resposiveness of gene expression. Endocrinology, 139:2048-2057.

Boskey A L, Maresca M, Doty S, Sabsay B and Veis A (1990). Concentration dependent effect of dentin-phosphophorin in the regulation of in vitro hydroxyapatite formation and growth. Bone Mineralisation, 11:55-65.

Boskey A L (1992). Mineral-matrix ineractions in bone and cartilage. Clinical Orthopaedics, 281:244-274.

Bredt D and Synder S (1990). Isolation of nitric oxide synthetase, a calmodulin-requiring enzyme. Proceedings of the National Academy of Sciences USA, 87:682-685.

Brown S D, Biddulph R B and Wilcox P D (1964). A strength-porosity relation involving different pore geometry and orientation. American Ceram Society Journal, 47:320

Brown E M, Gamba G, Riccardi D, Lombardi M, Butters R, Kifor O, Sun A, Hediger M A, Lytton J and Hebert S C (1993). Cloning and characterisation of an extracellular Ca^{2+} sensing receptor from bovine parathyroid. Nature, 366:575-580.

Buckwalter J A, Glimcher M J, Cooper R R and Recker R (1995). Bone biology part I. Journal of Bone and Joint Surgery, 77A:1256-1275.

Buckwalter J A, Glimcher M J, Cooper R R and Recker R (1996). Bone biology I: structure, blood supply, cells, matrix and mineralization. Inst course lect 45: 371-386.

Budal J (1979). The osteogenic capacity of the periosteum. Oral surgery, 47:227-229.

Burger E H and Klein-Nulend J (1999). Mechanotransduction inbone – role of lacuno-canalicular network. FASEB, 13:S101-S112.

Butler W T, (1989). The nature and significance of osteopontin. Conective Tissue Research, 23:123-136.

Calvo M S, Eyre D R and Gundberg C M (1996). Molecular basis and clinical application of biological markers of bone turnover. Endocrine Reviews, August:333-368.

Cameron J K, (1972). The Biochemistry and Physiology of Bone, (Ed) Bourne G H, 2nd Edition. (Academic Press, New York, USA).

Campbell (1987). Biology, 3rded, Benjamin/Cummings Publishing Co, Wokingham, UK.

Canalis E (1980). Effect of insulin-like growth factor I on DNA and protein synthesis in cultured rat calvaria. Journal of Clinical investigation, 66:709-719.
Canalis E (1986). Interlukin-1 has independent effects on deoxyribonucleic acid and collagen synthesis in cultures of rat calvariae. Endocrinology, 118:74-81.

Canalis E (1987). Effects of tumour necrosis factor on bone formation in vitro. Endocrinology, 121:1596-1604.

Carter D R, Wong M and Orr T E (1991). Musculoskeletal ontogeny, phylogeny, and functional adaptation. Journal of Biomechanics, 24:3-16.

Chen J, Shapiro H S and Sodek J (1992). Developmental expression of bone sialoprotein mRNA in rat mineralised connective tissues. Journal of Bone and Mineral Research, 7:987-997.

Cohen J and Harris W H (1958). The three-dimensional anatomy of haversian systems. The Journal of Bone and Joint Surgery 40/2:419-434.

Conn K M and Termine J D (1983). Matrix protein profiles in calf bone development. Bone, 6:33-36.

Cook M E, Petterson P H and Sunde M L (1984). Leg deformities: inability to increase severity by increasing body weight of chicks and poults. Poultry science, 63:620-627.

Cooke V E (2004). Growth associated and stress-induced mopathies in the domestic chicken (*Gallus domesticus*). Edinburgh University thesis.

Cooper R C and Cawley A J (1988). Blood supply to the periosteum of the canine tibia. American Journal of Veterinary Research, 49:1419-1423.

Creemers L B, Jansen D C, van Veen-Reurings A, van den Bos T and Everts V (1997). Microassay for the assessment of low levels of hydroxyproline. Biotechniques, 22:656-658.

Currey J D (1975). The effects of strain rate, reconstruction and mineral content on some mechanical properties of bovine bone. Journal of Biomechanics, 8:81-86.

Currey J D, Brear K and Zioupos P (1996). The effect of aging and changes in mineral content in degrading the toughness of human femors. Journal of Biomechanics, 29:257-260.

Damoulis P D and Hauscka P V (1997).nitric oxide acts in conjunction with proinflammatory cytokines to promote cell death in osteoblasts. Journal of Bone and Mineral Research, 12:412-422.

Day E J (1990). Future research needs to focus on new, old problems. Feedstuffs, 23:12-15.

Delany A M, Amling M, Priemal M, Howe C, Baron R and Canalis E (2000). Osteopenia and decreased bone formation in osteonectin-deficient mice. Journal of Clinical Investigation, 105:915-923.

Denhardt D T and Prince C W (1993). Extracellular and matrix proteins, (Ed) Kreis T and Vale R. (Oxford University Press, New York, USA)

Derkx P and Birkenhäger-Frenkel D H (1995). A thionin stain for visualizing bone cells, minerlizing fronts and cement lines in undecalcified bone sections. Biotechnic and Histochemistry, 70:70-74.

Dewhirst F E, Stashenko P P E, and Tsurumachi T (1985). Purification and partial sequence of human osteoclast –activating factor: Identity with interlukin 1 beta. Journal of Immunology, 135:2562-2568.

Dodds R A, Connor J R, James I E, Rykaczewski E L, Applebaum E, Dul E, and Gowen M (1995). Human osteoclasts, not osteoblasts, deposit OPN onto resorption surfaces, an in vitro and ex vivo study of remodelling bone. Journal of Bone and Mineral Research, 10:1666-1680.

Domenicucci M E, Goldberg H A, Hofmann T, Isenman D, Wasi S and Sodek J, (1988). Biochemistry Journal, 253:139-151.

Doty S B and Schofield B H (1976). Enzyme histochemistry of bone and cartilage cells. Prog Histomchemistry Cytochemistry, 8:1-38.

Ducy P, Zhang R, Geoffroy V, Ridall A L and Karsenty S (1997). Osf2/Cbfa1: a transcriptional activator of osteoblast differentiation. Cell, 89:747-754.

Ducy P, Starbuck M, Priemel M, Shen J, Pinero G, Geoffroy V, Amling M and Karsenty G (1999). A Cbfa1-dependent genetic pathway controls bone formation beyond embryonic development. Gene Development, 275:1025-1036.

Duxon M S, Flanigan TP, Reavly AC, Baxter GS, Blackburn T P and Fone K C (1997). Neuroscience, 76:323-329.

Einhorn T A (1996). The bone organ system; form and function. In: Osteoporosis, (Ed) Marcus R. (Academic Press, New York, USA).

Ellender G, Feik S A and Carach B J (1988). Periosteal structure and development in a rat caudal vertebra. Journal Anatomica, 158:173-187.

Ellis E S, Byrne C, Murphy O E, Tilford N S and Baxter G S (1996). Mediation by 5-hydroxytryptamine 2B receptors of endothelium-dependent relaxationinrat jugular vein. British Journal of Pharmacology, 114:400-404.

Engel J, Taylor W, Paulsson M, Sage H and Hogan B L M, (1987). Calcium binding domains and calium-induced conformational transition of SPARC/BM-40/osteonectin, an extracellular glycoprotein expressed in mineralised and nonmineralised tissues. Biochemistry 27:1483-1489.

Eriksen E F, Colvard D S, Berg N J, Graham M L, Mann K G, Spelsgerg T C and Riggs B L (1988). Evidence of estrogen receptors in normal human osteoblast-like cells. Science, 241:84-86.

Eyre-Brooke A L (1984). The periosteum: its function reassessed. Clinical orthopeadics, 198:300-307.

Farquharson C, Duncan A and Robins SP (1989). The effects of copper deficiency on the pyridinium crosslinks of mature collagen in the rat skeleton and cardiovascular system. Proceedings of the Society for Experimental Biology and Medicine 192:166-171.

Farquharson C and Loveridge N (1990). Cell proliferation within the growth plate of long bones assessed by bromodeoxyuridine uptake and its relationship to glucose 6-phosphate dehydrogenase activity. Bone and Mineral 10:121-130.

Farquharson C, Jefferies D (2000). Chondrocytes and longitudinal bone growth: The development of tibial dyschondroplasia. Poultry Science, 79:994-1004.

Farquharson C, Jefferies D, Seawright E and Houston B (2001). Regulation of chondrocytes terminal differentiation in the post embryonic growth plate: The role of PTHrP-Indian hedgehog axis. Endocrinology, 142:4131-4140.

Ferretti M, Muglia M A, Remaggi F, Canè V and Palumbo C (1999). Histomorphometric study on the osteocyte lacuno-canalicular network in animals of different species. II. Parallel-fibered and lamellar bones. International Journal of Anatomy and Embryology 104:121-131.

Fisher L W, Whitson S W, Avioli L V and Termine J D, (1983). Matrix sialoprotein of developing bone. Journal of Biological Chemistry , 285:12723-12727.

Fisher L W, Hawkins G R, Tuross N and Termine J D (1987). Purification and partial characterization of small proteoglycan I and proteoglycan-II, bone sialoprotein-I and sialoprotein-II, and osteonectin from the mineral compartment of developing human-bone. Journal of biological chemistry, 262:9702-9708.

Fisher L W, McBride O W, Termie J D and Young M F, (1990). Human-bone sialoprotein- deduced protein- sequence and chromosomal localisation. Journal of Biological Chemistry , 265:2347-2351.

Fisher L W (1993). Extracellular and matrix proteins, (Ed) Kreis T and Vale R. (Oxford University Press, New York, USA)

Folkman J and Moscona A (1978). Role of cell shape in growth control. Nature, 273:345-349.

Fox S w and Chow J W M (1998). Nitric oxide synthase expression in bone cells. Bone, 23:1-6.

Frost H M (1958a). Preparation of thin undecalcified bone sections by rapid manual method. Stain technology, 33:273-277.

Frost H M (1958b). Staining of fresh, undecalcified, thin bone sections. Stain technology, 135-146.

Frost H M (1964). Mathematical elements of lamellar bone remodelling. (Charles C Thomas, Springfield, IL).

Frost H (1969). Tetracycline-based histological analysis of bone remodelling. Calcified tissue research 3:211-237.

Frost H M (1973). Bone remodelling and its relationship to metabolic bone disease. (Ed)Charles Thomas. (Springfield, Illinois, USA).

Frost H M (1986). Intermediary organisation of the skeleton. (CRC Press).

Frost H M (1997). Defining osteopenias and osteoporosis: another view (with insights from a new paradigm). Bone, 20:385-391.

Fuji S, Honda S, Sekiya Y, Yamasaki M, Yamamoto M and Saijoh K (1998). Differential expression of nitric oxide synthase isoforms in form-deprived chick eyes. Current Eye Research, 586-593.

Gerstenfeld L C, Chipman S D, Glowacki J amd Lian J B (1987). Expression of differentiated function by mineralising cultures of chicken osteoblasts. Developmental Biology, 122:49-60.

Gerstenfeld L C, Riva A, Hodgens K, Eyre D R and Landis W J (1993). Post-translational control of collagen fibrillogenesis in mineralising cultures of chick osteoblasts. Journal of Bone and Mineral Research, 8:1031-1039.

Gilbert L, He X, Farmer P, Boden S, Kozolwski M, Rubin J and Nanes M S (2000). Inhibition of osteoblast differentiation by tumour necrosis factor-α. Endocrinology, 141:3959-3964.

Giraud-Guille M M (1988). Twisted plywood architecture of collagen fibrils on human compact bone osteons. Calcified Tissue International 42:167-180.

Glimer M J (1989). Mechanism of calcification: role of collagen fibrils and collagen-phosphoprotein complexes in vitro and in vivo. Anatomical record, 224:139-153.

Glusa E and Roos A (1996). Endothelial 5-HT receptors mediate relaxation of porcine pulmonary arteries in response to ergotamine and dihydroergotamine. British Journal of Pharmacology, 119:330-334.

Goddard C, Wilkie R s and Dunn I C (1988). The relationships between insulin-like growth factor-1, growth hormone, thyroid hormones and insulin in chickens selected for growth. Domestic Animal Endocrinology, 5:165:176.

Gotoh Y, Gerstenfeld L C and Glimer M J (1990). Identification and characterisation of the major chicken bone phosphoprotein- analysis of its synthesis by cultured embroyonic chick osteoblasts. European Journal of Biochemistry, 187:49-58.

Gratzner H G 91982). Monoclonal antibody to 5-Bromo- and 5-Io-dodeoxyuridine: Anew reagent for detection of DNA replication. Science 218:474-475.

Green M R and Pastewka J V (1974). Simultaneous differential staining by a cationic carbocyanine dye of nucleic acids, proteins and conjugated proteins. II. Carbohydrate and sulphated carbohydrate-containing proteins. Journal of Histochemistry. Cytochemistry, 22:774-781.

Gruber H E and Mekikian P (1991). Application of stains-all for demarcation of cement lines in methacrylate embedded bone. Biotechnic and Histochemistry, 66:181-184.

Gruber H E and Stasky A A (1997). Large specimen bone embedment and cement line staining. Biotechnic and Histochemistry, 72:198-201.

Hagenaars J F, Van der Fraan A M, Kawilaring-de Haas E M, Visser J M and Nijweide P J., (1989). Osteoclast formation from cloned pluripotent hemopoietic stem cells. Bone and Mineral, 6:170-189.

Hall T J and Kenny A D, (1985). Role of carbonic anhydrase in bone resorption induced by 1,25-dihydroxyvitamin D3 in vitro. Calcified Tissue International, 37:134-142.

Hall TJ and Chambers T J, (1990). Na/H antiporter is the primary proton transport system used by osteoclasts during bone resorption. Journal of Cell Physiology, 142:420-424.

Ham A W and Cormack D H, (1979). Histology, 8th Edition. (J B Lippincott Co, Philadelphia USA).

Hansson L, Stinstrom A and Thorngren K (1974). Diurnal variation of longitudinal bone growth in the rabbit. Acta Orthop Scand, 45:499-507.

Hardingham T E and Fosang A J, (1992). Proteoglycans- many forms and many functions. FASEB J, 6:861-870.

Harms G, van Goor H, Koudstaal J, de Ley L, and Hardonk M J (1986). Imminuohistochemical demonstration of DNA-incorporated 5-bromodeoxyuridine in frozen and plastic embedded sections. Histochemistry, 85:139-143.

Hauschka P V, Lian J B, and Gallop P M (1975). Direct identification of the calcium-binding amino acid, gamma-carboxyglutamic-acid containing protein from bone. Proceedings of the National Academy of Sciences USA, 73:3925-3929.

Haussler M, Nagode L A and Rasmussen H (1970). Induction of intestinal brush border alkaline phosphatase by vitamin D and identity with Ca-ATPase. Nature, 228:1199-1201.

Hernandez C J, Majeska R J and Schaffler M B (2004). Osteocyte density in woven bone. Bone, 35:1095-1099.

Herring G M (1972). The Biochemistry and Physiology of Bone, (Ed) Bourne G H. 2nd edition. (Academic Press, New York, USA)

Hert J, Pribylova E and Liskova M (1972). Microstructure of compact bone after intermittent loading. Acta Anatomica 82:218-230.

Hock J M, Centralla M and Canalis E (1988). Insulin-like growth factor I (IGF-I) has independent effects on bone matrix formation and cell replication. Endocrinology, 122:254-260.

Hofbauer L C, Dunstan C R, Spelsberg T C, Riggs B L, and Khosla S (1998). Osteoprotegrin production by human osteoblast lineage cells is stimulated by vitamin D, bone morphometric protein-2, and cytokines. Biochemistry and Biophysics Research Communication, 250:776-781.

Hofbauer L C, Lacey D L, Dunstan C R, Spelsberg T C, Riggs B L and Khosla S (1999). Interlukin-1 beta and tumour necrosis factor-α, but not interlukin-6, stimulate osteoprotegrin ligand gene expression in human osteoblast cells. Bone, 25:255-259.

Hofbauer L C, Shui C, Riggs B L, Dunstan C R, Spelsberg T C, O'Brein T and Khosla S (2001). Effects of immunosuppressants on receptor activator of NF-κB ligand and osteoprotegrin production by human osteoclastic and coronary artery smooth muscle cells. Biochemistry and Biophysics Research Communication, 280:334-339.

Hollmann M and Heinmann S F (1994). Cloned glutamate receptors. Annual Review in Neuroscience, 17:31-108.

Hoyer D, Clarke D E, Fozard J R, Hartig PR, Martin G R, Mylecharane E F, Saxena P R and Humphrey P P (1994). Pharmacology Rewiew, 46:157-203.

Hudson H A, Britton W M, Rowland G N and Buhr R J (1993). Histomorphometric bone properties of sexually immature and mature white leghorn hens with evaluation of fluorochrome injection on egg production traits. Poultry science, 72:1537-1547.

Hunter G K and Goldberg H A (1993). Nucleation of hydroxyapatite by bone sialoprotein. Proceedings of the National Academy of Sciences USA, 90:8562-8665.

Hunter G K, Kyle C L and Goldberg H A (1994). Modulation of crystal formation by bone phosphoproteins: structural specificity of the osteopontin-mediated inhibition of hydroxyapatite formation. Biochemical Journal, 300:723-728.

Ibaraki K, Whitson S W, Termine J D and Young MF (1992). Bone matrix mRNA expression in differentiating fetal bovine osteoclasts. Journal of Bone and Mineral Research, 7:743-754.

Iozzo R V and Murdock A D, (1996). Proteoglycans of the extracellular environment: Clues from the gene and protein side offer novel perspectives in molecular diversity and function. FASEB J, 10:598-614.

Jarvinen T L, Pajamaki I, Sievanen H, Vouhelainen T, Tuukkanen J, Jarvinen M and Kannus P, (2003). Femoral neck response to exercise and subsequent deconditioning in young and adult rats. Journal of Bone and Mineral Research, 18:1292-1299.

Johansson S, Kjellen L, HookM and Timpl R, (1981). Substrate adhesion of rat hepatocytes- a comparison of laminin and fibronectin as attachment proteins. Journal of cell biology, 90:260-264.

Jordan G, Loveridge N, Bell K L, Power J and Reeve J (2000). Spatial clustering of remodelling osteons in the femoral neck cortex: A cause of weakness in hip fracture?. Bone, 26:305-313.

Jundt G, Berghauser K-H, Termine J D and Schulz A (1987). Osteonectin-a differentiation marker of bone cells. Cell Tissue Research, 248:409-415.

Kanwar Y, Veis A, Kimura J and Jakubowski (1984). De novo cellular synthesis of sulphated ptoyeoglycans of the developing renal glomerulus in vivo. Proceedings national academic science, USA, 81:762-766.

Kaye M, (1984). When is it an osteoclast?. Journal of Clinical Pathology, 37:398-400.

Kee N, Sivalingam S, Boonstra R and WojtowiczJ M (2002). The utility of BrdU as proliferative markers of adult neurogenesis. Journal of neuroscience Methods, 115:97-105.

Kestin SC, Knowles TG, Tinch AE, Gregory NG (1992). Prevalence of leg weakness in broiler chickens and its relationship with genotype. Veterinary Record, 131:190-19

Kimmel D B and Jee W S S, (1977). Research in radiobiology, Annual report of work in progress in the International Irradiation Program. (Radiology Laboratory, University of Utah, College of Medicine C00.119.252).

Kirkwood J K, Spratt D M J and Duignan P J (1989). Patterns of cell proliferation and growth rate in limb bones of the domestic fowl (*Gallus domesticus*). Research in Veterinary Science, 47:139-147.

Kitaoka K, Furman B and Saha S (1998) periosteum: its biomechanical role in bone fracture. North American Congress of Biomechanics, Waterloo, Ontario, Canada, Aug 14-18.

Klein D C and Raisz L G (1970). Prostaglandins: stimulation of bone resorption in tissue culture. Endocrinology, 86:1436-1440.

Kleinman H K, Klebe R J and Martin G R (1981). Role of collagenous matrices in the adhesion and growth of cells. Journal of Cell Biology, 88:473-485.

Klein-Nulend J, van der Plas A, Semeins C M, Ajubi N E, Frangos J A, Nijweide P J and Burger E H (1995a). Sensitivity of osteocytes to biomechanical stress in vitro. FASEB, 9:441-445.

Klein-Nulend J, Semeins C M, Ajubi N E, Nijweide P J, and Burger E H (1995b). Biochemistry Biophysics Research Communication, 217:640-648.

Kojima T, Leone C, Marchildon G, Marcum J and Rosenberg R, (1992). Isolation and characterisation of heparan-sulfate proteoglycans produced by cloned rat microvascular endothelial cells. Journal of biochemistry, 267:4858-4869.
Komm B S, Terpening C M, Benz D J, Graeme K A, O'Malley B W and Haussler M R (1988). Estrogen binding receptor mRNA and biologic response in osteoblast-like osteosarcoma cells. Science, 241:81-84.

Komori T, Yagi H, Nomura S, Yamaguchi A, Sasaki K, Deguchi K, Shimizu Y, Bronson R T, Gao, Y-H, Inada M, Sato M, Okamoto R, Kitamura Y, Yoshiki S and Kishimoto T (1997). Target disruption of Cbfa 1 results in a complete lack of born formation owing to maturational arrest of the osteoblasts. Cell, 89:755-764.

Kuykindoll R J, Nishimura H, ThomasonD B and Nishimoto S K (1999). Osteopontin expression in spontaneously developed neointima in fowl (*Gallua Gallus).* The Journal of Experimental Biology, 203:273-282.

Lanyon L E and Bourn S (1979). The influence of mechanical function on the developemant and remodelling of the tibia: An experimental study in sheep. The Journal of Bone and Joint Surgery, 61:263-273.

Lanyon L E (1980). The influence and function on the development of bone curvature. An experimental study on the rat tibia. Journal Zool London, 192:457-466.

Lanyon L E (1992). Bone biology and the skeletal disorders in poultry. Whitehead C C (Carfax publishing Co, Abingdon,UK).

Lanyon L (1993). Osteocytes, strain detection, bone modelling and remodelling. Calcified Tissue International, 53:102-107.

Lee SL and Glimer M J (1981). Purification, composition and p-31 NMR spectroscopic properties of a non-collagenous phosphoprotein isolated from chicken bone matrix. Calcified tissue international, 33:385-394.

Leivo I, Vaheri A, Timpl R and Wartiovaara J, (1980). Appearance and distribution of collagens and laminin in the early mouse embryo. Developmental Biology, 76:10-114.

Leivo I and Wartiovaara J (1982). Embryonic development (eds Burger and Webb). A R Liss, New York, USA.

Leivo I, Alitalo K, Risteli L, Vaheri A, Timpl R and Wartiovaara J, (1982). Basal lamina glycoproteins laminin and type-IV collagen are assembled into a fine fibred matrix in cultures of a teratocarcinoma-derived endodermal cell line. Experimental cell research, 137:15-23.
Lerner U H (1996). Principles of Bone Biology, (Academic Press, San Diego, USA).

LeTerrier C, Rose N, Constantin P and Nys Y (1998). Reducing growth rate of broiler chickens with a low energy diet does not improve cortical bone quality. British Poultry Science, 39:24-30.

Lian J B, Stein G S, Bortell R and Owen T A (1991). Phenotype suppression: A postulated molecular mechanism for mediating the relationship of proliferation and differentiation by fos/jun interactions at AP-1 sites in steroid responsive promoter elements of tissue-specific genes. Journal of Cell Biochemistry, 45:9-14.

Lian J B and Stein G S (1996).Osteoblast Biology. In: Osteoporosis, (Ed) MarcusR. (Academic Press, New York, USA).

Liggett W H, Lian J B, Greenberger J S and Glowacki J (1994). Osteocalcin promotes differentiation of osteoclast progenitors from murine long-term bone marrow cell cultures. Journal of Cell Biochemistry, 55:190-199.

Lim S K, Won Y J, Lee H C, Huh K B and Park Y S (1999). A PCR analysis of ERα and ERβ mRNA abundance in rats and the effect of ovariectomy. Journal of Bone Mineral Research, 14:1189-1196.

Liu W, Toyosawa S, Furuich T, KanataniN, Yoshida C, Liu Y, Himeno M, Narai S, Yamaguchi A and Komori T (2001). Overexpression of Cbfa1 in osteoblasts inhibits maturation and causes osteopenia with multiple fractures. Journal of Cell Biology, 155:157-166.

Lodish H, Baltimore D, Berk A, Zipursky S L, Matsudaira P and Darnell (1996). Molecular and Cell Biology. (3rd edition) Scientific American Books Inc, New York, USA.

Lorenzo J A, Sousa S L, Alander C, Raisz L G and Dinarello C A(1987). Comparison of the bone-resorbing activity in the supernatants from phytohemagglutinin-stimulated human peripheral blood mononuclear cells with that of cytokines through the use of an antiserum to interlukin 1. Endocrinology, 121:1164-1170.

Lynch M P, Stein J L, Stein G S and Lian J B (1994). Apoptosis during in vitro bone formation. Journal of Bone and Mineral Research, 9:S352.

Macpherson H, Noble B S and Ralston S H (1999). Expression and functional role of nitric oxide synthase isoforms in human osteoclast-like cells. Bone, 24:179-185.

Manson J D and Waters N E (1965). Observations on the rate of maturation of the cat osteon. Anatomy London, 99:539-549.

Marcelli C, Yates A J and Mundy G R (1990). In vivo effects of human recombinant transforming growth factor β on bone turnover in normal mice. Journal of Bone and mineral Research, 5:1087-1096.

Mark M P, Butler W T, Prince C W, Finkelman R D and Ruch J V (1988). Developmental expression of 44-kDa bone phosphoprotein (osteopontin) and bone γ-carboxyglutamic acid (GLA)-containing protein (osteocalcin) in calcifying tissues of rat. Differentiation, 37:123-136.

Marks S C and Popoff S N (1988). Bone cell biology: the regulation of development structure and function in the skeleton. American Journal of Anatomy 183:1-44.

Martin R B (1984). Porosity and specific surface of bone. CRC Critical Review of Biomedical Engineering, 10:179-222.

Martin R B and Ishida J (1989). The relative effects of collagen fibre orientation, porosity, density and mineralisation on bone strength. Journal of Biomechanics, 22:419-426.

Martin R B and Boardman D L (1993). The effects of collagen fibre orientation, porosity, density and mineralisation on bovine cortical bone-bending properties. Journal Biomechanics, 26:1047-1054

Martin R B, Burr D B and Sharkey N A (1998). Skeletal tissue mechanics. (Springer-verlag, New York, USA)

.

Martin R B (2000). Does osteocyte formation cause the nonliear refilling of osteons?. Bone, 26:71-78.

Martin T J, Findley D M and Moseley J M (1996). Peptide hormones acting on bone. In: Osteoporosis, (Ed) MarcusR. (Academic Press, New York, USA).

Marotti G and Zallone A Z (1980). Changes in the vascular network during the formation of haversian systems. Acta anat 106:84-100.

Marroti G I, Ferritti M, Remaggi F and Palumbo C (1995). Quantitative evaluation on osteocyte canalicular density in human secondary osteons. Bone, 16:125-128.

Mason D J, Suva L J, Genever P G, Patton A J, Stueckle S, Hillam R A and Skerry T M (1997). Mechanically regulated expression of a neural glutamate transporter in bone. A role for excitatory amino acids as osteotropic agents?. Bone, 29:199-205.

Mason I F, Taylor A, Williams J G, Sage H and Hogan B L M, (1986). Evidence from molecular-cloning that SPARC, a major product of mouse embryo parietal endoderm is related to anendothelial-cell culture shock glycoprotein of MR-43000. Embryology Journal, 5:1465-1472.

Mcvey J H, Nomura S, Kelly P, Mason I J and Hogan B L M, (1988). Characterisation of the mouse SPARC/osteonectin gene intron exon organisation and an unusual promoter region. Journal of Biological Chemistry, 263:6958-6965.

Miller S C and Jee W S S (1987). The bone lining cell: A distinct phenotype?. Calcified tissue international 41:1-5.

Miller S C and Jee W S S (1992). Bone lining cells. Bone vol 4 (ed, Hall B K) CRC press, Boca Raton.
Miller S S, Wolf A M and Arnaud C D (1976). Bone cells in culture: morphological transformation by hormones. Scirncr, 192:1340-1343.

Milligram J W (1977). Radiographical and pathological assessment of the activity of pagents' disease of bone. Clinical orthopaedic related research, 127:43-54.

Miyazaki Y, Setoguchi M, Yoshida S, Higuchi Y,Akizuki S and Yamamoto S, (1990). Journal of Biological Chemistry, 265:14432-14438.

Moran R, Darzynkiewiez Z, Staiano-Coico L and Melamed M R (1985). Detection of 5-bromodeoxyuridine (BrdUrd) incorporation by monoclonal antibodies : role of DNA denaturation step. J histochem cytochem 33:821-827.

Morstyn G, Hsu S M, Kinsella T, Gratzner H G, Russo A and Mitchell J B (1983). Bromodeoxyuridine in tumours and chromosomes detected with a monoclonal antibody. Journal of clinical investigation 72:1844-1850.

Morton L F, Fitzsimmons C M, Reuterberg J and Barnes M J (1987). Platelet-reactive sites in collagen- collagen-I and collagen-II possess different aggregatory sites. Biochemical Journal, 248:483-487.

Mundlos S, Schwahn B, Reichert T and Zabel B (1992). Distribution of osteonectin mRNA and protein during human embryonic and fetal development. Journal of Histochemistry Cytochemistry, 40:283-291.

Mundy G R, (1999). Bone remodelling. Primer on the Metabolic Bone Diseases and Disorders of Mineral Metabolism, 4th edition. (Ed) Favus M J (Lippincott Williams and Wilkins, New York, USA).

Nakase T, Takaoka K, Hirakawa K, Hirota S, Takemura T, Onoue H, Takebayashi K, Kitamura Y and Nomura S (1994). Alterations in the expression of osteonectin, osteopontin and osteocalcin mRNAs during the development of skeletal tissues in vivo. Bone and mineral, 26:109-122.

Nakata K, Nakahara H,Kimura T, Kojima A, Iwasaki M, Caplan A and Ono K (1992). Collagen gene expression during chondrogenesis from chick periosteum-derived cells. FEBS, 299:278-282.

Neville A C (1984). Cuticle: Organisation. In Biology of the Integument. I. Invertebrates, (Ed) Bereiter-Hahn J, Matoltsy A G and Richards K S. (Springer-Verlag, Berlin, Germany).

Newbury J W, Baksi N, Dhillon A S, Zimmerman N G, Truitt S G, and Riedinger (1988). Histomorphometry and vitamin D metabolism of valgus-valgus deformity in broiler chickens. Aivan Diseases, 32:704-712.

Nijweide p J, van der Plas A, Alblas M J and Klein-Nuland J (2003). Osteocyte isolation and culture; osteoblast mix. In, Bone research Protocols.(Ed) Helfrich M P and Ralston S H, (Humana Press, New Jersey USA).

Noble B S, Stevens H, Loveridge N and Reeve J (1997). Identification of apoptotic changes in osteocytes in normal and pathological human bone. Bone 3:273-282.

Noble B S, Stevens H Y, Peet N M, Reilly G, Currey J D and Skerry T M (1998). Matrix microdamage and osteocytes apoptosis: a mechanism for targeting bone resorption. Bone, 23(supplement 5) 179 (abstract).

Noble B S and Reeve J (2000). Osteocytes function, osteocyte death and bone fracture resistance. Molecular Cell Endocrinology, 159:7-13.

Noda M, and Denhardt D T (2002). In; Principles of Bone Biology 2nd edition. (Eds) Bilezikian J P, Raisz L G and Rodan G A. (Acadenic Press, London, UK).

Nurminskaya M, Magee C, Faverman L and Linsenmayer T F (2003). Chondrocytes-derived transglutaminate promotes matyration of preosteoblasts in periosteal bone. Developmental Biology, 263:139-152.

Ogawa E, Maruyama M, Kagoshima H Inuzuka M, Lu J, Satake M, Shipesada K and Ito Y (1993). PEBP2/PEA2 represents a family of transcription factors homologous to the products of the drosophila runt gene and the human AML1 gene. Proceedings of the National Academy of Sciences USA, 90:6859-6863.

Oldberg A, Jirskog-Hed B, Axelsson S and Heinegard D (1989). Regulation of bone sialoprotein mRNA by steroid hormones. Journal of Cell Biology, 109:3183-3186.

Oldenberg A, Franzen A, Heingard D, Pierschbacher M and Ruoslahti E, (1988). Identification of a bone sialoprotein receptor in osteosarcoma cells. Journal of Biological Chemistry , 263:19433-19436.

Onoe R O C, Miyaura C,Ohta H, Nozawa S, and Suda T (1997). Expression of estrogen β in rat bone. Endocrinology, 138:4509-4512.

Osbody P C, Rothe L, Bekker S, Anderson F and Osbody P (2000). Decreased nitric oxide levels stimulate osteoclasrogenesis and bone resorption both in vitro and in vivo on the chick chorioallantoic membrane in association with neoangiogenesis. Jouranl of Bone and Mineral Research, 15:474-488.

Otto F, Thronlkl A P, Crompton T, Denzel A, Gilmour K C., Rosewell I R, Stamp G W H, Beddington R P, Nundlos S, Olsen B R, Selby P B and Owen M J (1997). Cbfa1, a candidate gene for cleidocranial dysplasia syndrome, is essential for osteoblast differentiation and bone development. Cell, 89:765-771.

Oursler M J, Osbody P, Pyfferon J, Riggs B L and Spelsberg T C (1991). Avian osteoclasts as estrogen target cells. Proceedings of the National Academy of Sciences USA, 88:6613-6617.

Owen M, (1963). Cell population kinetics of an osteogenic tissue 1. Journal of Cell Biology, 19:19-32.

Owen M, (1978). Histogenesis of bone cells. Calcified Tissue Research, 25:205-207.

Owen T A, Aronow M, Shalhoub V, Barone L M, Wilming L, Tassinari M S, Kennedy M B, Pockwinse S, Lian J B and Stein G S (1990). Progressive development of the rat osteoblast phenotype in vitro: Reciprocal relationships in expression of genes associated with osteoblast proliferation and differentiation during formation of the bone extra cellular matrix. Journal of Cell Physiology, 143:420-430.

Owen T A, Aronow M S, Barone L M, Bettencourt B, Stein G S and Lian J B(1991). Pleiotropic effects of vitamin D on osteoblast gene expression are related to the proliferative and differentiated state of the bone cell phenotype: dependency upon basal levels of gene expression, duration of exposure, and bone matrix competency in the normal rat osteoblast culture. Endocrinology, 128:1496-1504.

Parfitt A M (1983). The physiologic and clinical significance of bone histomorphometric data. Bone histomorphometry techniques and interpretation (edited by R R Recker). CRC Press, Boca Raton, USA.

Parfitt A M, Matthews C H E, Villanueva A R, Kleerekopper M, Frame B, and Rao DS (1983). Relationships between the surface, volume and thickness of iliac trabecular bone in ageing and in osteoporosis; implications for the microanatomic and cellular mechanisms of bone. Journal of Clinical Investigations, 72:1396-1409.

Parfitt A M, (1984). The cellular basis of bone remodelling: the quantum concept re-examined in the light or recent advances in the cell biology of bone. Calcified Tissue International, 36:S37-S45.

Parfitt A M (1987). Bone and plasma homeostasis. Bone (New York, USA)

Patterson P H, Cook M E, Crenshaw T D, and Sunde M L (1986). Mechanical properties of the tibiotarsus of broilers and poults loaded with artificial weight and fed various dietary protein levels. Poultry Science, 65:1357-1364.

Patton A J, Genever P G, Birch M A, Suva L J and Skerry T M (1998). Expression of an N-Methyl-D-Aspartate-Type receptor by human and rat osteoblasts and osteoclasts suggests a novel glutamate signalling pathway in bone. Bone, 22:645-649.

Pead M J, Suswillo R, Skerry T M, Vedi S and Lanyon L E (1988). Increased 3H uridine levels in osteocytes following a single short period of dynamic loading in vivo. Calcified tissue international 43:92-97.

Pease D C (1960). Proceedings of the 4th international conference on electron microscopy, 2:139-159.

Pistillides A A, Rawlinson S C F, Suswillo R F L, Bourrin S Zaman G, and Lanyon L E (1995). Mechanical strain induced NO production by bone cells: a possible role in adaptive bone (re)modelling. FASEB Journal, 9:1614-1622.

Pitsillides A A, Rawlinson S C F, Mosley J R, and Lanyon L E (1999). Bone's early responses to mechanical loading differ in distinct genetic strains of chick: Selection for enhanced growth reduces skeletal adaptability. Journal of Bone and Mineral Research, 14:980-987.

Pollock J, Forstermann U, Mitchell J, Warner T and Murad F(1991). Putification and characterisation of particulate endothelium-derived relaxing factor synthase from cultured and native bovine aortic endothelial cells. Proceedings of the National Academy of Sciences USA, 88:10480-10484.

Portigiatti-Bardos M, Bianco P and Ascenzi A (1983). Distribution of osteonic and interstitial components in the human femoral shaft with reference to structure, calcification and mechanical properties. Acta Anatomica, 115:178-186.

Postlethwaite A E, Seyer J M and Kang A H (1978). Chemotactic attraction of human fibroblasts to type I,II, and III collagens and collagen –derived peptides. Proceedings of National Academic Science USA, 75:871-875.

Poulos P, Reiland S, Elwinger K and Olsson S, (1978). Skeletal lesions in the broiler with special reference to dyschondroplasia (osteochondrosis). Pathology frequency and clinical significance in two strains of birds and low energy feed. Acta radiol 358:229-276.

Price P A and Baukol S A (1980). 1α,25-dihydroxyvitamin D_3 increases synthesis of the vitamin K-dependent bone protein by osteosarcoma cells. Journal of Biological Chemistry, 255: 11660-11663.

Prockop D J, Kivirkko K I, Tuderman L and Guzman N A, (1979). The biosynthesis of collagen and its disorders. New England Journal of Medicine, 301:13-23-77-85.

Quarles L D, Yohy D A, Lever L W, Caton R and Wenstrup R J (1992). Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. Journal of Bone and Mineral Research, 7:683-692.

Ragsdale B D, Madewell J E and Sweet D E (1981). Radiologic and pathologic analysis of solitary bone lesions. Part II, periosteal reactions. Radiol clin North Amer, 19:749.

Raisz L G, Fall P M, Gabbitas B Y, NcCarthy T L, Kream B E, and Canalis E (1993). Effects of prostaglandin E2 on bone formation in cultured fetal rat calvariae: role os insulin-like growth factor-I. endocrinology, 133:1504-1510.

Raouf A and Seth A (2000). Ets transcription factors and targets in osteogenesis. Oncogene, 19:6455-6463.

Rapraeger A, Krufta A and Olwin B B, (1991). Requirement of heparan-sulphate for BFGF- mediated fibroblast growth and myoblast differentiation. Science, 252:1705-1708.

Rawlinson S C F, Haj E I, Minter S L, Tavares I A and L E Lanyon (1991). Load related increases of prostaglandin production in cores of adult canine cancellous bone in vivo – a role for prostacyclin in adaptive bone remodelling. Journal of Bone and Mineral Research 6:1345-1351.

Recker R (1983). Bone histomorphometry: techniques and interpretation. Boca Raton, Florida: CRC Press Inc.

Reddy R P (1996). Symposium: The effects of long term selection on growth of poultry. Poultry Science, 75:1164-1167.

Reinholt F P, Hultenby K, Oldberg A, and Heinegrd D (1990). OPN: a possible anchor of osteoclasts to bone. Proceedings of the National Academy of Sciences USA, 87:4473-4475.

Remaggi F, Canè V, Palumbo C and Ferretti M (1998). Histomorphometric study on the osteocyte lacuno-canalicular network in animals of different species. I. Woven-fibred and parallel-fibered bones. International journal of anatomy and embryology 103:145-155.

Rhinelander F W (1974). Tibial blood supply in relation to fracture healing. Clinical orthopedics, 105:34-81.

Rhinelander F W and Wilson J W (1982). Blood supply to developing, mature and healing bones. In Summer-Smith G ed. Bone in clinical orthopedics. (WB Sanders Co, Philadelphia, USA)

Rickard D, Harris S A, Turner R, Khosla S and Spelsberg T C (2002). In; Principles of Bone Biology 2nd edition. (Eds) Bilezikian J P, Raisz L G and Rodan G A. (Academic Press, London, UK).

Riggs C M, Lanyon L E and Boyde A (1993a). Functional association between collagen fibre orientation and locomotor strain direction in cortical bone of the equine radius. Anatomical Embryology, 187:231-238.

Riminucci M, Bradbeer J N, Corsi A, Gentili C, Descalzi F, Cancedda R and Bianco P (1998). Vis-aVis cells and the priming of bone formation. Journal of Bone and Mineral Research, 13:1852-1861.

Riggs C M, Vaughan L C, Evans G P, Lanyon L E and Boyde A (1993b). Mechanical implications of collagen fibre orientation in cortical bone of the equine radius. Anatomical Embryology, 187:239-248

.

Robey P G, Bianco P and Termine J D (1992). The cellular bilogy and molecular biochemistry of bone formation. In, Disorders of mineral matebolism. (Eds), Coe F L and Favus M F, (Raven Press, New York, USA)

Robins S P (1988). Functional properties of collagen and elastin. Balliere's Clinical Rheumatology, 2:1-36.

Romanowski R, Jundt G, Termine J D, von der Mark K and Schulz A (1990). Immunoelectron microscopy of osteonectin and type I collagen in osteoblasts and bone matrix. Calcified Tissue International, 46:353-360.

Rosenberry H H, Hastings A B and Morse J K (1931). X-ray analysis of bone and teeth. Journal of Biological Chemistry 90:395-407.

Rubin C T and Lanyon L C (1987). Osteoregulatory nature of mechanical stimuli: function as a determinant for adaptive remodelling in bone. Journal of Orthopaedic Research, 5:300-310.

Ruoslahti E (1981). Fibronectin. Journal of Oral Pathology, 10:3-13.

Ruoslahti E, and Pierschbacher M D, (1987). New perspectives in cell-adhesion-RGD and integrins. Science 238:491-497.

Sage H, Johnson C and Bornstein P, (1984). Characterisation of a novel serum albumin-binding glycoprotein secreted by endothelial-cells in culture. Journal of Biological Chemistry, 259:3993-4007.

Saksela O, Moscatelli D, Sommer A and Rifkin D, (1988). Endothelial cell-derived heparan-sulfate binds basic fibroblast growth-factor and protects if from proteolytic degradation. Journal of Cell Biology, 107:743-751.

Sandhu H and Jande S (1981). Radioisotope and morphometric evaluation of the effects of β-aminopropionitrile on chick bone matrix formation and its minerlization. Acta anat, 111:281-288.
Schaffler M B and Burr D B (1988). Stiffness of compact bone: effects of porosity and density. Journal of Biomechanics, 21:13-16.

Schinke T and Karsenty G (2002). In; Principles of Bone Biology 2nd edition. (Eds) Bilezikian J P, Raisz L G and Rodan G A. (Acadenic Press, London, UK).

Schwartz S m and Biewener A A (1992). Shape and scaling. In, Biomechanics, structures and systems. (Ed), Biewener A A. (Oxford University Press, UK).

Silve C M, hradek G T, Jones A L and Arnaud C D (1983). Parathyroid hormone receptor on intact embryonic chicken bone: characterisation and cellular isolation. Journal of Cell Biology, 94:379-386.

Simonet W S, Lacey D L, Dunstan C R, Kelly M, Chang M S, Luthy R, Nguyen H Q, Woden S, Bennett L, Boone T, Shimamoto G, DeRose M, Elliott R, Colombero A, Tan H L, Trail G, Sullivan J, Davy E, Bucay N, Renshaw-Gegg L. Hughes T M, Hill D, Patison W, Campbell P, Sander S, Van G, Tarpley J, Gerby P, Lee R and Boyle W J (1997). Osteoprotegrin: A novel secreted protein involved in the regulation of bone density. Cell, 89:309-319.

Skedros J G, Bloedaum R D, Manson M W and Bramble D M (1994). Analysis of a tension/compression skeletal system: Possible strain-specific differences in the hierarchical organisation of bone. The Anatomical Record, 239:396-404.

Skerry T M, Bitensky L, Chayen J and Lanyon L E (1989). Early strain related changes in enzyme activity in osteocytes following bone loading in vivo. Journal of Bone and Mineral Research, 4:783-788.

Skerry T M and Genever P G (2001). Glutamate signalling in non-neuronal tissues. TRENDS in Pharmacological Sciences, 22:174-181.

Smith E, Frenkel B, Schlegel R, Giordano A, Lian J B, Stein J L and Stein G S (1995). Expression of cell cycle regulatroty factors in differentiating osteoblasts: post-proliferative upregulation of cyclins B and E. Cancer Research, 55:5019-5024.

Sobel A E and Honok A (1948). Calcification of teeth I. composition in relation to blood and diet. Journal of Biological Chemistry 176:1103-1122.

Somerman M J, Fisher L W, Foster R A and Sauk J J, (1988). Human-bone sialoprotein-I and sialoprotein-II enhance fibroblast attachment invitro. Calcified Tissue International, 43:50-53.

Squier C A, Ghoneim S, and Kremenak (1990). Ultrastructure of the periosteum from membrane bone. Journal Anatomica, 171:223-239.

Stegeman H and Stadler K (1967). Determination of Hydroxyproline. Clinica Chimica Acta 18:267-273.

Stein G S, Lian J B and Owen T A (1990). Relationship of cell growth to the regulation of tissue-specific gene expression during osteoblast differentiation. FASEB 4:3111-3123.

Stein G S and Lian J B (1995). Molecular mechanisms mediating proliferation/differentiation interrelationships during progressive development of the osteoblast phenotype: update 1995. Endocrinology Review, 4:290-297.

Stover S M, Pool R R, Martin R B and Morgan J P (1992). Histologic features of the dorsal cortex of the third metacarpal bone mid-diaphysis postnatal growth in thoroughbred horses. Journal of Anatomy 181:455-469.

Strauss P G, Closs EI, Schmidt J, and Erfle V (1990). Gene expression during osteogenic differentiation in mandibular condyles in vitro. Journal of Cell Biology, 110:1369-1378.

Suda T, Takahashi N and Martin T J (1992). Modulation of osteoclast differentiation. Endocrine Review, 13:66-80.

Takeuchi Y, Fukumoto S, Nakayama K and Matsumoto T (1994). Decrease in transforming growth factor-β receptors by differentiation of osteoblastic cells is dependent upon collagen production. Journal of Bone and Mineral Research, 9:S126.

Tapp C (1966). Tetracycline labelling methods of measuring the growth of bones in the rat. Journal of Bone and Joint Surgery. 48B:517-525.

Terai K, Takano-Yamamoto T, Ohba Y, Hiura K, Sugimoto M, Sato M, Kawahata H, Inaguma N, Kitamura Y and Nomura S (1999). Role of OPN in bone remodelling caused by mechanical stress. Journal of Bone and Mineral research, 14:839-849.

Termine J D, Kleinman H K, Whitson W S, Conn K M, McGarvey M L and Martin G R, (1981). Osteonectin, a bone-specific protein linking mineral to collagen. Cell, 26:99-105.

Thitunavukkarasu K, Mahajan M, McLarren K W, Stifani S and Karsenty G (1998). Two domains unique to osteoblast-specific transcription factors Osf2/Cbfa1 conttribute to its transactivation function and its ability to heterodimerise with CBFβ. Molecular Cell Biology, 18:4197-4208.

Thompson D D (1980). Age changes in bone mineralization, cortical thickness and Haversian canal area. Calcified Tissue International, 31:5-11.

Thomson B M and Loveridge N, (1992). Bone growth In: the control of fat and lean deposition.(Ed), Buttery P J, Boorman K N, Lindsay D B, (Butterworth-Heinemann, Oxford, UK)

Thorp B H and Waddington D (1997). Relationships between the bone pathologies, ash and mineral content of long bones in 35-day-old broiler chickens. Research in Veterinary Science, 62:67-73.

Timpl R, Rohde H, Robey P G, Rennard S I, Foidart J M and Martin G R (1979). Laminin—a glycoprotein from basement membranes. Journal of Biochemistry, 254:9933-9937.

Tsuji K, Ito Y and Noda M (1998). Expression of the PEBP2alphaA/AML3/CBFA1 gene is regulated by BMP4/7 heterodimer and its overexpression suppresses type I collagen and osteocalcin gene expression in osteoblastic and nonosteoblastic mesenchymal cells. Bone, 22:87-92.

Tsukii K, Shima N, Mochizuki S, Yamaguchi K, Kinosuki M, Yano K, Shibata O, Udayawa N, Yasuds H, Suda T and Hiyashio K (1998). Osteoclast differentiation factor mediates an essential signal for bone resorption induced by 1 alphs,25-dihydroxyvitamin D3, prostaglandin E2, or parathyroid hormone in the microenviroment of bone. Biochemistry, Biophysical Research Communications, 246:337-341.

Turner C H, Forwood M R and Otter M W (1994). Mechanotransduction in bone: do cells act as sensors of fluid flow?. FASEB Journal, 8:875-878.

Turner C H and Pavalko (1998). Mechanostransduction and functional response of the skeleton to physical stress: the mechanisms and mechanics of bone adaptation. Journal of Orthopeadic Science, 3:346-355.

Uchida A, Kikuchi T and Shimomura Y (1988). Osteogenic capacity of cultured human periosteal cells. Acta orthop scand, 59:29-33.
Uchiyama A, Suzuki M, Lefteriou B, and Glimer M J (1986). Biochemistry, 25:7472-7583.

Udagawa N, Takahashi N and Akatsu T, (1990). Origin of osteoclasts: mature monocytes and macrophages are capable of differing into osteoclasts under a suitable microenviroment prepared by bone marrow derived stromal cells. Proceedings of the National Academy of Sciences USA, 87:7260-7264.

Uhtoff H K and Jaworski Z F G (1978). Bone loss in response to long-term immobilisation. Journal of Bone Joint Surgery. 60B:420-429.

Ullmer C, Schmuck K, Kalkman H O and Lubbert H (1995). Expression of serotonin receptor mRNAs in blood vessels. FEBS Letters, 370:215-221.

Vaes G, (1988). Cellular biology and biochemical mechanism of bone resorption. A review of recent developments on the formation,activation and mode of action of osteoclasts. Clinical Orthopaedics, 231:239-271.

Van der Meulen M CH, Beaupre G S and Carter D R (1993). Mechanobiologic influences in long bone cross-sectional growth. Bone, 14:635-642.

Van't Hof R J, Armour K J, Smith L M, Armour K E, Wei X Q, Liew F Y and Ralston S H (2000). Requirement of the inducible nitric-oxide synthase pathway for IL-1 induced osteoclastic bone resorption. Proceedings of the National Academy of Sciences USA, 97:7993-7998.

Vaughan J (1981). The physiology of bone. 3rd edition (Clarendon Press, Oxford)

Vitorvic D, Nikolic Z and Cvetkovic D(1995). In vivo tetracycline labelling as a measure of rearing-system influence on chicken-bone dynamics. Anat Histol Embryol, 24:85-86.

VlodavskyI and Gospodarowiez D (1981). Respective roles of laminin and fibronectin inadhesion of human carcinoma and sarcoma cells. Nature, 289:304-306.

Voet D and Voet J G (1995). Biochemistry, 2nd edition. (John Wiley and Sons Ltd, Chichester, UK).

Wartiovaara J, Leivo I and Vaheri A (1980). The cell surface: mediator of developemental processes. 38th Symposium of the Society for Developmental Biology.

Wasi S, Otsuka K, Yao K L, Tung P S, Aubin J E, Sodek J and Termine J D, (1984). An osteonectinlike protein in porcine periodontal ligament fibroblasts. Journal of Biological Chemistry, 62:470-478.

Westbroek I, van der Plas A, de Rooij K E, Klein-Nulend J and Nijweide J (2001). Expression of serotonin receptors in bone. Journal of Biological Chemistry, 276:28961-18968.

Williams B, Solomon S, Waddington D, Thorp B and Farquharson C (2000a). Skeletal development in the meat-type chicken. British Poultry Science, 41:141-149.

Williams B, Waddington D, Solomon S and Farquharson C (2000b). Dietary effects on bone quality and turnover. Research in Veterinary Science, 69:81-87.

Williams B G, Waddington D, Murray D H and Farquharson C. (2004) Bone strength during growth: Influence of growth rate on cortical porosity and mineralization. Calcified Tissue International 74 (3):236-245

Wise D (1970). Comparison of the skeletal system of growing broiler and laying strain chickens. British Poultry Science, 333-339.

Wlodarski K H (1989). Normal and heterotopic periosteum. Clinical Orthopaedics, 241:265-277.

Wojcik (1999). Expression of PTHrP and the PTH/PTHrP receptor in purified alveolar epithelial cells, myoepithelial cells, and stromal fibroblasts derived from the lactating rat mammary gland. Experimental Cell Research, 248:415-442.

Wolff J (1892). Das gesetz des transformation der knochen. Berlin, Hirechwald.

Xie A, Cho H, Calaycay J, Munford R, Swiderek K, Lee T, Ding A, Troso Y and Nathan C (1992). Cloning and characterisation of inducible nitric oxide synthase from mouse macrophages. Science, 259:225-228.

Yamaguchi D T, Ma L, Lee A, Huang J, and Gruber H E, (1994). Isolation and characterisation of gap junctions in the osteoblastic MC3T3-E1 cell lines. Journal of Bone Mineral Research, 9:791-803.

Yamanda K M and Olden K (1978). Fibronectins-adhesive glycoproteins of cell surface and blood. Nature, 275:179-184.

Yamazuki K, Suzuki M, Mikuni-Tagagaki Y, Hiraiwa K, Lefteriou B and Glimer M J (1989). Preparation of monoclonalantibodies to chicken bone phosphoproteins. Calcified tissue international, 43:41-43.

Yanagishita M and Hascall V C (1992). Cell surface heparan sulphate proteoglycans. Journal of Biochemistry, 267:9415-9454.

Yeni Y N, Brown C U and Norman T L (1998). Influence of bone composition and apparent density on fracture toughness of the human femur and tibia. Bone, 22:79-84.

Young M F, Kerr J M, Termine J D, Wewer U M, Wang M G, McBride O W and Fisher L W, (1990). cDNA cloning, messenger –RNA distribution and heterogeneity, chromosomal location, and RFLP analysis of human osteopontin (OPN). Genomics, 7:491-502.

Zaman G, Pistillides A A, Rawilson S C, Suswillo R f, Mosley J R, Cheng M Z, Platts L, Hukkanen M, Polak J M and Lanyon L E (1999). Mechanical strain stimulates rapid, transient increases in nitric oxide release from eNOS in bone cells but does not effect eNOS mRNA expression. Journal of Bone and Mineral Research, 14:1123-1131.

Zohar R, Lee W, Arora P, Cheifetz S, McCulloch C and Sodek J (1997). Single cell analysis of intracellular osteopontin in osteogenic cultures of fetal rat calvarial cells. Journal of Cellular Physiology, 170:88-100.

Appendix

Ascorbic Acid

Ascorbic acid	7.225	mg
Culture medium	5.0	ml

TBS

Tris 7.6	50	mM
NaCl	150	mM

RIPA buffer

component	stock	dilution	In 50mls
20mM Tris 8	1M	1/50	1 ml
137mM NaCl	5M	1/36.5	1.37mls
10% Glycerol	100%	1/10	5mls
1% Igepal	100%	1/100	0.5mls
0.1% SDS	20%	1/200	0.250mls
0.5% Nadeoxycholate	5%	1/10	5mls
2mM EDTA	0.5M	1/250	0.2mls

Make up to 50mls , and take 10mls plus 1 Complete protease inhibitor tablet for working solution.

Toluidine blue O

Toluidine blue O	0.1%	w/v
PO_4 buffer	5.5	pH

RNase free paraffin embedded tissue– by hand

Day 1	carried out at 4°C		
	70% ethanol	1x	30 mins
	80% ethanol	2x	30 mins
	95% ethanol	2x	30 mins
	leave at room temp overnight. Ensure set up wax to melt overnight.		
Day 2	rotate in fume hood where possible		
	100% ethanol	2x	1 hr
	Xylene	2x	1 hr
	Wax	2x	1hr
	Embed		

Complete culture medium (cCM)

DMEM		420 ml
Gentimicin	0.5%	2.5 ml
FBS	10%	50 ml
Pyruvate	1%	5 ml
Fungizone	1%	5 ml
Ascorbic acid	35µl/ml	17.5 ml

Bone chip- digestion solution

Trypsin/EDTA solution (0.25% trypsin + 0.1% EDTA)
Diluted 50:50 with DMEM

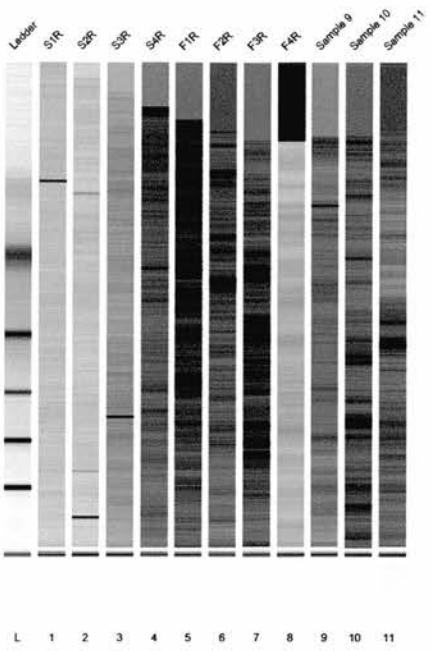
Trypsin/EDTA dilution	1ml
Collagenase	3.2mg
PBS	4ml

Table A1 Methodology of agilent analysis of RNA extracted from fast and slow growing bones using LCM

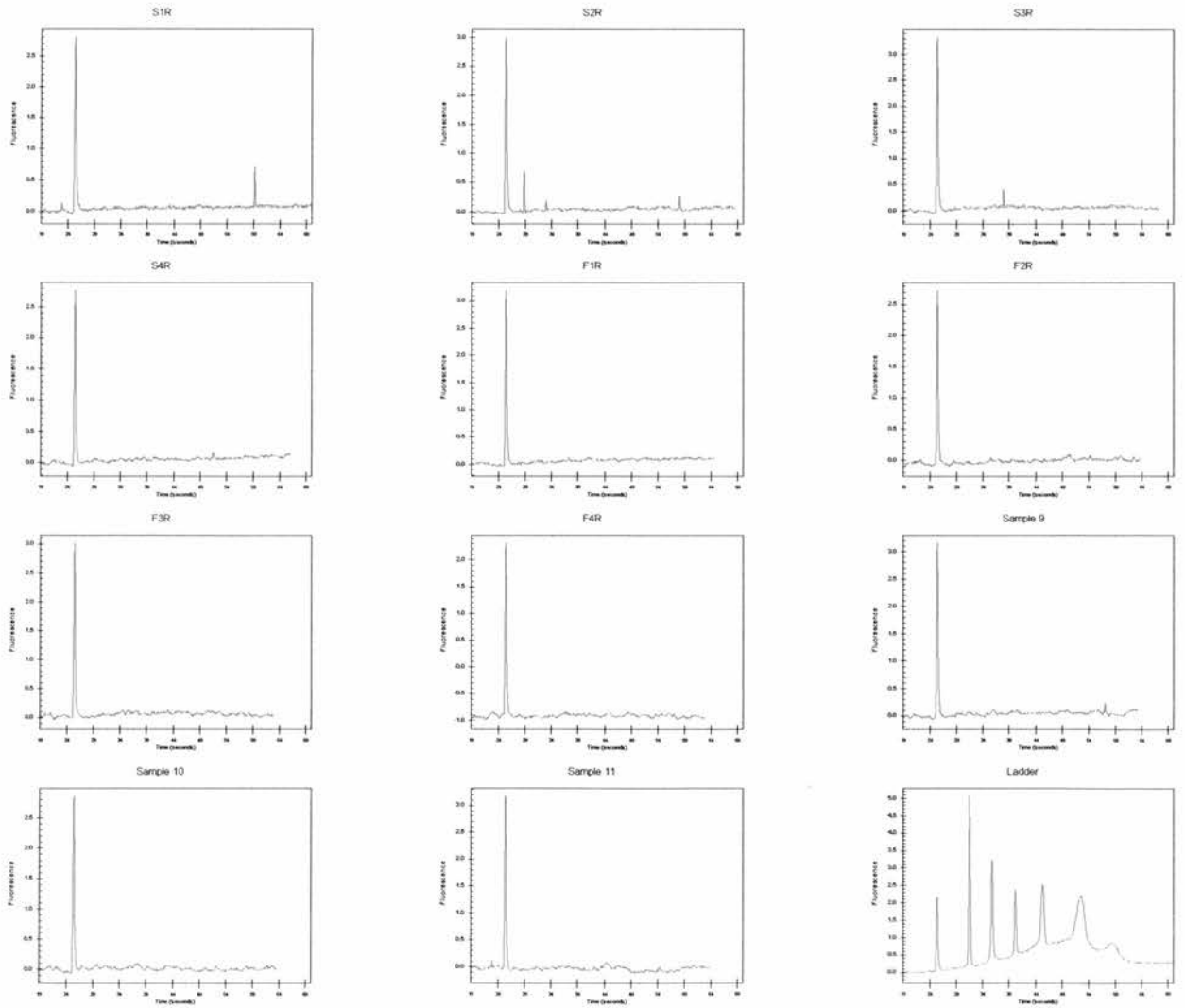
Agilent analysis	Bone sample fast	Bone sample fast	Bone sample fast	Bone sample fast	Bone sample fast	Bone sample fast	Bone sample fast	Bone sample fast	Bone sample slow	Bone sample slow	Bone sample slow	Bone sample slow	Bone sample slow	Bone sample slow
1st analysis	F1 R	F1 L	F2 R	F2 L	F3 R	F3 L	F4 R	F4 L	S1 R	S1 L	S2 R	S2 L	S3 R	S3 L
2nd analysis	F1 R+L		F2 R+L		F3 R+L		F4 R+L		S1 R+L		S2 R+L		S3 R+L	
3rd analysis	F1+2+3+4								S1+2+3					

Assay: Eukaryote Total RNA Pico
Data Path: \\Gho-rinetapp1a\IGF_Lab_Bk\Agilent2100\Bio Sizing\Data

Read: 17/06/2004 16:11:05 (A.02.12 SI292)
Modified: 17/06/2004 16:39:06 (A.02.12 SI292)

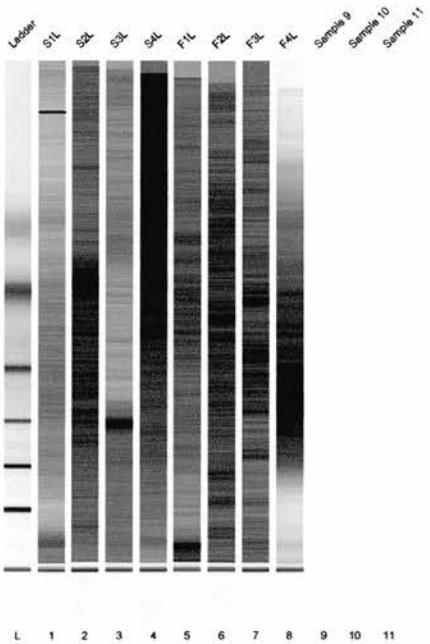


Instrument:	G2938A, Serial# DE11400907, Firmware Version A.01.16		
Assay:	C:\Program Files\Agilent 2100 Bioanalyzer\Bio Sizing\Assays\RNA\Eukaryote Total RNA Pico.asy		
Title:	Eukaryote total RNA Pico		
Version:	1.0		
Comments:	Copyright 1999 - 2001 Caliper Technologies Corp.		
	1.0 Initial Release		
Ladder Concentration:	1000 pg/ul	Baseline:	19 Seconds
Min Peak Height:	0.5 (above baseline)	Filter Width:	0.5 Seconds
Slope Threshold:	0.2 /Second	Baseline Plateau:	0.5 Seconds
Min Peak Width:	0.5 Seconds	Polynomial Order:	6
Start Time:	20 Seconds		
End Time:	69 Seconds		
Notes:			

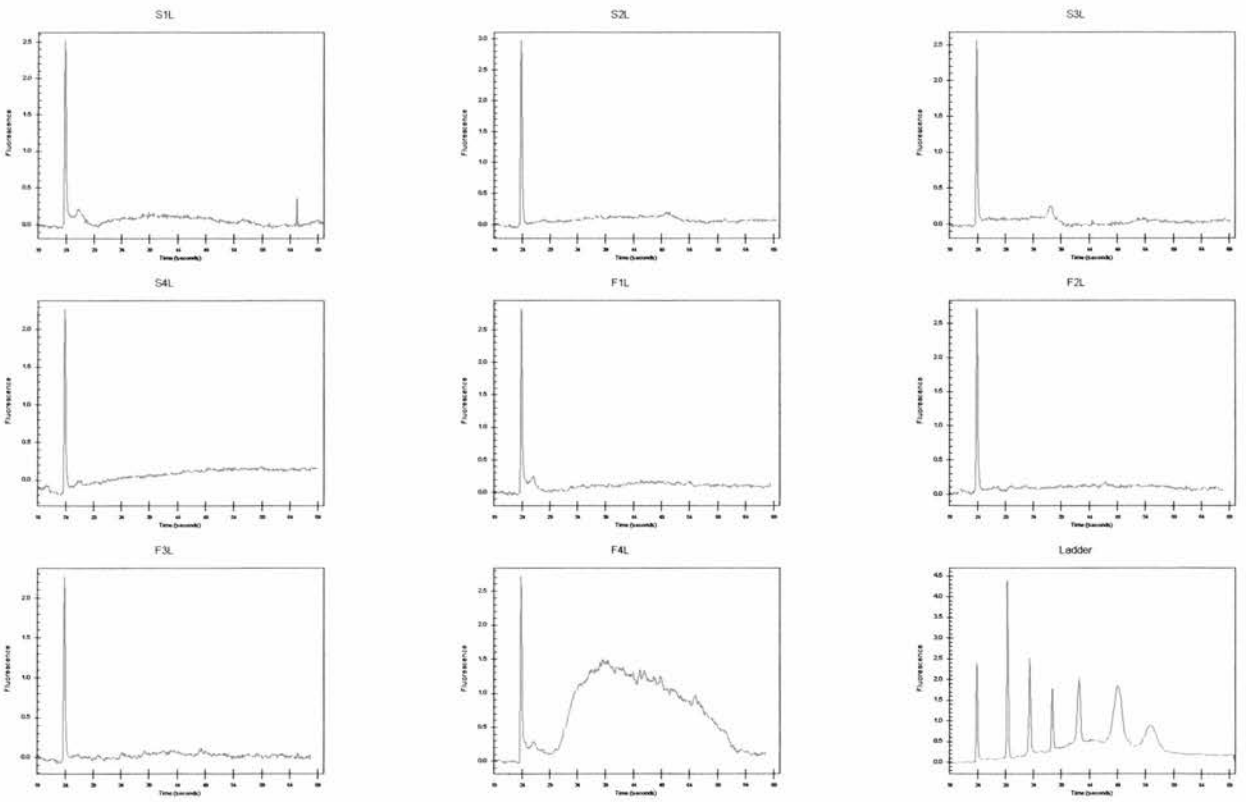


Assay: Eukaryote Total RNA Pico
Data Path: \\Gho-rinetapp1a\IGF_Lab_Bk\Agilent2100\Bio Sizing\Data

Read: 18/06/2004 12:09:58 (A.02.12 SI292)
Modified: 18/06/2004 14:09:22 (A.02.12 SI292)

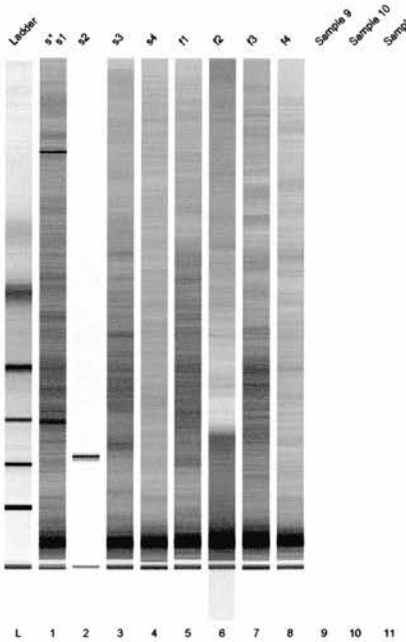


Instrument:	G2938A, Serial# DE11400907, Firmware Version A.01.16		
Assay:	C:\Program Files\Agilent 2100 Bioanalyzer\Bio Sizing\Assays\RNA\Eukaryote Total RNA Pico.asy		
Title:	Eukaryote total RNA Pico		
Version:	1.0		
Comments:	Copyright 1999 - 2001 Caliper Technologies Corp.		
	1.0 Initial Release		
Ladder Concentration:	1000 pg/ul	Baseline:	19 Seconds
Min Peak Height:	0.5 (above baseline)	Filter Width:	0.5 Seconds
Slope Threshold:	0.2 /Second	Baseline Plateau:	0.5 Seconds
Min Peak Width:	0.5 Seconds	Polynomial Order:	6
Start Time:	20 Seconds		
End Time:	69 Seconds		
Notes:			

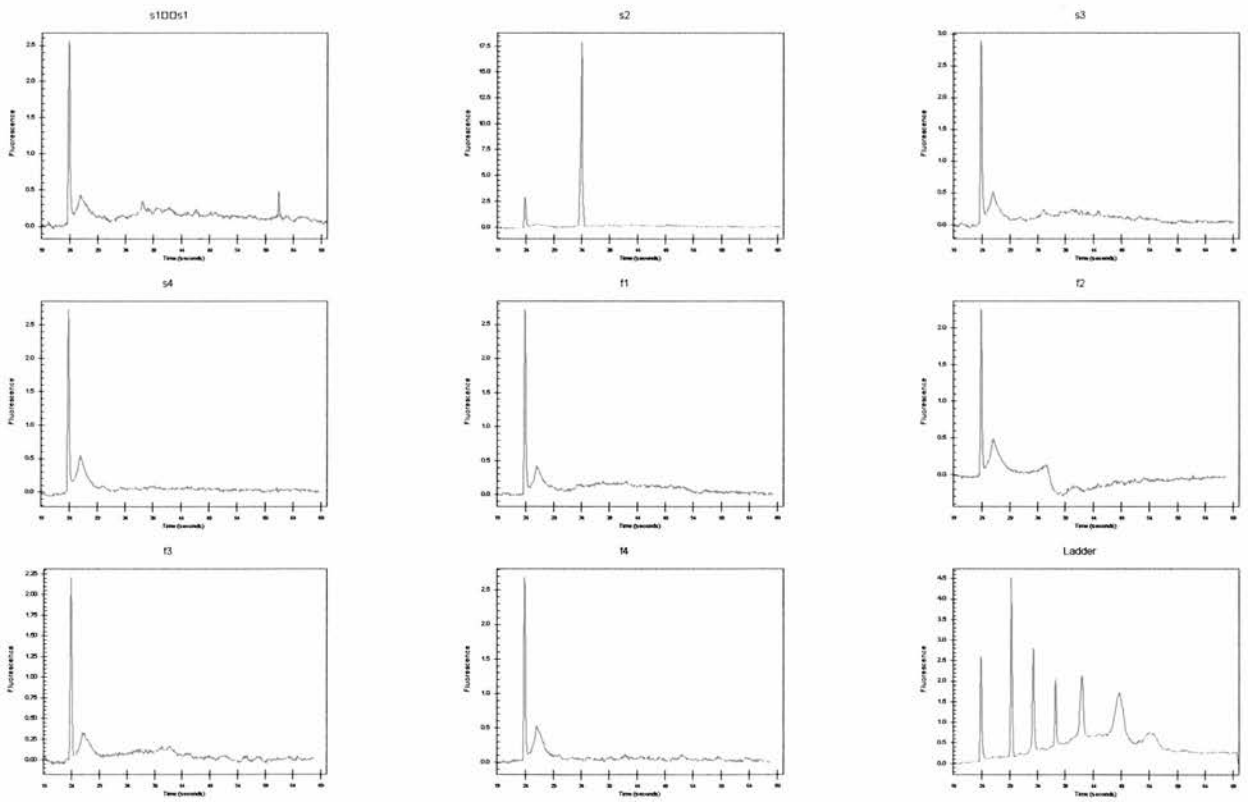


Assay: Eukaryote Total RNA Pico
Data Path: \\Gho-rinetapp1a\IGF_Lab_Bk\Agilent2100\Bio Sizing\Data

Read: 22/06/2004 10:50:09 (A.02.12 SI292)
Modified: 22/06/2004 11:20:17 (A.02.12 SI292)



Instrument:	G2938A, Serial# DE11400907, Firmware Version A.01.16		
Assay:	C:\Program Files\Agilent 2100 Bioanalyzer\Bio Sizing\Assays\RNA\Eukaryote Total RNA Pico.asy		
Title:	Eukaryote total RNA Pico		
Version:	1.0		
Comments:	Copyright 1999 - 2001 Caliper Technologies Corp.		
	1.0 Initial Release		
Ladder Concentration:	1000 pg/ul	Baseline:	19 Seconds
Min Peak Height:	0.5 (above baseline)	Filter Width:	0.5 Seconds
Slope Threshold:	0.2 /Second	Baseline Plateau:	0.5 Seconds
Min Peak Width:	0.5 Seconds	Polynomial Order:	6
Start Time:	20 Seconds		
End Time:	69 Seconds		
Notes:			



Calcif Tissue Int (2004) 74:236–245
DOI: 10.1007/s00223-002-2124-0

Calcified Tissue International

Bone Strength During Growth: Influence of Growth Rate on Cortical Porosity and Mineralization

B. Williams,[1] D. Waddington,[2] D. H. Murray,[1] C. Farquharson[1]

[1]Bone Biology Group, Division of Integrative Biology, Roslin Institute, Scotland
[2]Division of Genetics and Biometry, Roslin Institute, Scotland

Received: 20 August 2002 / Accepted: 23 May 2003 / Online publication: 2 October 2003

Abstract. Although it is well accepted that bone architecture adapts to withstand the loads placed on it, the manner in which this occurs in the immature growing skeleton is not fully understood. To investigate the possible mechanisms, we have compared morphometric differences between tibiae from chickens with fast and those with slow growth potential and also distinguished between the effects of genetic potential and growth rate on their impact on bone quality. Two different fast-growing (*ad lib* modern) strains, one additionally feed-restricted and one slow-growing (control) strain of chicken, were compared at 15 and 42 days of age. The *ad lib* modern strains had similar final body weights and were approximately twice the weight of the control and restricted-fed birds. Tibiae from the control and restricted birds had a higher ash content and lower porosity than the *ad lib* modern strain at 42 days. The porosity was a result of rapid primary osteon formation at the periosteal surface and incomplete infilling of the resultant canal by osteoblasts. When adjusted to average body weight of contemporaries, bones from the control strain and the restricted-fed modern birds were stiffer and at least as strong as those from the fast growing *ad lib*-fed birds. In conclusion, rapid bone deposition at the periosteal surface was associated with decreased mineralization, increased cortical porosity, and altered biomechanical properties. Our results also indicate that growth rate, and not genetic potential, of the fast growing birds was responsible for the rapid periosteal bone deposition.

Key words: Bone — Mineralization — Porosity — Growth — Mechanical properties

The deleterious effects of rapid body growth on skeletal strength have recently been recognized [1]. This has implications for both animals and humans but is of particular significance and concern in economically important agricultural species. Genetic selection for muscle growth has resulted in increased demands on skeletal integrity, and skeletal failure in the modern broiler (meat-type) bird, primarily associated with rapid longitudinal and circumferential growth of long bones, is an increasing welfare and economic issue. To date, research has been predominantly concerned with growth plate cartilage and disorders of longitudinal bone growth such as rickets and tibial dyschondroplasia [2]. In contrast, relatively little data exist on the underlying mechanisms that result in a high incidence of cortical bone fractures [3] although a recent study has investigated the possible effect of growth rate on skeletal parameters of the meat-type bird [4].

As first proposed in Wolff's law [5] bone architecture of both the mature and growing skeleton adapts to withstand the extremes of functional load-bearing [6–8]. In response to increased loads during growth, bones enlarge their circumference, thereby increasing their cross-sectional moment of inertia (CSMI) and consequently their bending strength, by the rapid laying down on the periosteal surface of newly formed primary bone [9]. In the broiler chick this primary bone takes the form of circumferential lamellae that are orientated parallel to the bone surface with incorporated primary osteons [10, 11]. The development of primary osteons within circumferential lamellar bone occurs by altered osteoblastic activity and differential bone growth, resulting in longitudinal depressions on the periosteal surface. The eventual entrapment of blood vessels and periosteal osteoblasts and the subsequent infilling of the canals by concentric laminae of bone results in the formation of the primary osteon [12].

We and others have previously reported that throughout the productive life-span, cortical bone from a heavy modern strain of bird was consistently more porous and less well-mineralized than that from a slower growing control strain [1, 11]. It is unclear, however, if this cortical bone morphology observed in the modern broiler is directly related to the rapid radial expansion of load-bearing bones necessary to withstand the rapid increases in body weight. In addition, it is also uncertain

Correspondence to: C. Farquharson; E-mail: colin.farquharson@bbsrc.ac.uk

Table 1. Experimental design

Bird group	No. of birds in whole experiment*	No. of birds blood sampled and culled at day 15**	No. of birds blood sampled and culled at day 42**
Modern (restricted)	60	18	18
Modern (*ad lib*)	60	18	18
Commercial	60	18	18
Control	60	18	18

*Birds of each group were divided into 3 separate pens each containing 20 birds. Each pen was within 1 of 3 blocks each containing 1 pen from each group
**Six birds per pen were sampled

whether the increased porosity observed is due to increased bone resorption within an osteon remodelling unit and the possible formation of super-osteons as observed in human bone [13, 14] or a reduced bone production rate in the infilling process during primary osteon formation.

The present study aimed to confirm the differences previously observed in bone mineralization and porosity between modern fast-growing and control, slow-growing meat-type strains of chicken at 15 and 42 days old [1] and to make preliminary investigations on the mechanical properties of bone. In addition, some of the possible mechanisms of the increased porosity observed in the modern strains of chickens were examined. Groups of two genetically distinct lines of modern, fast growing birds, and a control, slow growing strain were grown on *ad libitum feeding*. In addition, birds from one of the modern lines were placed on a restricted feeding regime to grow at a similar rate to the control birds. Combinations of these treatments allow the effects of genetic potential and growth rate to be separated, in their impact on the quality of diaphyseal cortical bone of the chick tibiotarsus.

Materials and Methods

Birds, Housing and Feeding Regimes

Three strains of bird were used: a current fast-growing commercial strain, a modern fast-growing strain undergoing selection for growth performance and skeletal health, and a slow-growing control strain that has not been subject to selection since 1972, labelled, respectively, commercial, selected, and control in this study. Sixty male day-old chicks of the control and commercial strains, and 120 of the selected strain were placed into 12 pens. The pens were grouped into 3 blocks, each block containing 1 pen each of the commercial and control strains, and 2 of the selected strain. Each pen contained 20 birds (Table 1). During the first week the pens had 23 hours light and 1 hour dark per day, and 21 hours light and 3 hours dark per day thereafter. For the first 4 days, all birds were fed *ad lib* on a standard broiler starter diet. From 5 days of age, one pen of selected strain birds in each block (Table 1) was fed at 50% of the *ad lib* consumption rate on a diet that contained double the Ca and P content, giving a daily Ca and P intake similar to that of the *ad lib*-fed selected strain birds (*Ad lib*: Ca 1.1%, available P 0.5%, restricted: Ca 2.0%, available P 1.0%.) All birds were switched to finisher diets at 15 days of age. The restricted-fed selected birds continued to be fed at 50% of the *ad lib* consumption rate on a finisher diet that again compensated for Ca and P intake. (*Ad lib:* Ca 1.0%, available P 0.45%, restricted: Ca 2.0%, available P 0.9%). In the absence of previous *ad lib* intake data on the selected strain, the daily feed intake was initially based on data from the *ad lib* food intake of a similar strain of fast-growing broiler. *Ad lib* food intake in the selected strain was monitored on a weekly basis, and the restricted pens' rations for the subsequent week were adjusted where *ad lib* food intake was found to be greater than expected.

Blood and Bone Mineral Status. At 15 and 42 days of age, six randomly chosen birds per pen were blood sampled, then culled by cervical dislocation, weighed, and both tibiotarsi were removed (Table 1). Blood ionized Ca content was measured using a Ciba-Corning 634 Ca^{++}/pH analyser. Plasma total Ca and inorganic P concentration were measured using commercially available kits (Wako Chemicals GmbH, Neuss, Germany). Segments from the proximal third of the right tibiotarsus shafts were analyzed for bone ash, Ca and P content as previously described [1].

Bone Dimensions and Mechanical Properties. The left tibiotarsi were x-rayed, and total bone and marrow width at approximately 30 points in the mid-diaphyseal segment were measured using NIH image, and then averaged as previously described [15]. CSMI were calculated assuming tibia to be circular. Breaking strengths were determined by three-point destructive bending. The tests were carried out with a Lloyd Instruments LRX Universal materials testing machine fitted with a 2500 Newton load cell and running the *Nexygen 2.2* software package between two 10 mm diameter supports, set 30 mm apart, with the center of the bone aligned with the breaking probe. The 10 mm diameter crosshead breaking probe approached the bone at 30 mm min^{-1} until the tibiotarsi was broken. The breaking strength was determined as the maximum load (N) to failure. Tibiotarsi stiffness (N/mm) was determined automatically from the slope of the load-displacement curve. To avoid introducing additional variation in strength and stiffness, each bone was oriented to ensure that bending occurred around the posterior-anterior axis.

Porosity Analysis and Cement Line Detection. Histological samples of mid-diaphyseal segments from the right tibiotarsi of birds were prepared for porosity analysis as previously described [1]. Measurements were made separately, at ×20 magnification, for endosteal and periosteal areas that were defined as the area of bone within 80 μm of the endosteal or periosteal surfaces, respectively. It was recognized from previous studies [1] that the size and shape of the primary osteons was not evenly distributed around the periosteum. This is likely to reflect differences in circumferential growth rates which will vary depending on whether the cortex was under tension or compression due to the natural bending of the tibiotarsus. Therefore, four fields of view on each of 3 sections (100 μm apart) were analyzed for each bone area (anterior, posterior, medial

and lateral) for each bird, and the average value was calculated. For the detection of cement lines, which mark the location of the bone surface during the reversal period in the formation of secondary osteons, serial sections were stained with 0.1% w/v toluidine blue O in phosphate-citrate buffer (pH 5.5) for 20 sec at RT. After thorough rinsing in water and dehydration the sections were mounted in DePeX.

Enzyme Histochemistry. The distal shaft segments from the right tibiotarsi were immersed in a hexane freezing bath [16] and 10 μm-thick frozen sections were cut on a microtome housed in a Brights OT cryostat (Huntington, UK). After air-drying, unfixed samples were reacted for alkaline phosphatase (ALP) and tartrate-resistant acid phosphatase (TRAP) activity using established techniques [16].

Statistical Analysis

The combination of strains and feeding regimes included 4 bird groups: *ad lib* control, commercial, selected, and restricted-fed selected. Data were analyzed by split-plot analysis of variance, fitting block and pen as random factors, to examine differences between bird groups, ages and their interaction. Comparisons between bird groups were split into those between the three *ad lib* fed groups (selected, commercial and control), and those due to restricted feeding (selected *ad lib* versus restricted) and their average values over the 2 ages were assessed against the variation between pens. Age comparisons, and age by bird group interactions, were assessed against within pen variation. Statistical comparisons therefore, as appropriate, have different estimates of standard error and error degrees of freedom. Pooled standard errors derived from the analysis of variance, and on the scale of the analysis, are presented throughout. Data were logarithmically transformed where necessary to equalize variation over treatments. Body weight, bone width, CSMI, stiffness, maximum load before rupture and plasma inorganic P were analyzed on the natural log scale, and summarized by back-transformed (geometric) means unless otherwise stated.

The logarithms of tibiotarsus strength measurements show a strong linear relationship with the logarithm of body weight, and analyses of these variables were therefore repeated with log (body weight) as an additional covariate. However, the slope of the relationship differs at each age, and so bone strength comparisons among the 4 bird groups, corrected for body weight, could only be made for birds of the same age.

Results

Body Weight

Within the *ad lib* birds, at 15 days the two modern strains (selected strain = 439 g, commercial strain = 400 g) were considerably heavier than the control strain at 222 g. By 42 days, the selected and control strains were about 5.5 times as heavy (selected = 2433 g, control = 1226 g), but the commercial strain was 6.4 times as heavy at 2556 g (SEM of log weight = 0.026; strain.age interaction $F_{2,126} = .98$, $P < 0.01$). At both ages, restricted feeding reduced body weight in the selected strain by about 40% (15 days 276 g, 42 days 1424 g; SEM of log weight = 0.024; $F_{1,6} = 217.64$, $P < 0.001$), but it still remained higher than the control birds by about 20%.

Bone Growth

The total width of the tibiotarsus in the heavier modern strains was greater at 15 days (selected = 3.65 mm, commercial = 3.48 mm) than the control strain at 2.74 mm. However, width increased proportionately 10% faster with age in the commercial strain than the selected and control strains (selected 7.16 mm, commercial 7.51 mm, control 5.36 mm; SEM of log width 0.021; strain.age interaction $F_{2,126} = 3.48$, $P < 0.05$). Restricted feeding reduced bone width by about 20% in the selected strain at both ages (15 days = 2.96 mm, 42 days = 5.5 mm; SEM = 0.013; $F_{1,6} = 164.52$, $P < 0.001$), to about 5% greater than the control strain.

Differences in log (CSMI) have the same pattern as those in log (bone width), as the variables have a correlation of 0.99. Restricted feeding of the selected strain lowered CSMI values by 60% ($F_{1,6} = 94.24$; $P < 0.001$), to a level 35% greater than the control strain. The relationship of the logarithms of CSMI and body weight is shown in Figure 1. A separate linear relationship, common to the four groups, is necessary at each age: the lines have a common slope, but the intercept for the 15-day birds is higher than that for the 42-day birds ($t_{139} = 6.43$; $P < 0.001$). There is no evidence for different relationships between bird groups within each age group. The patterns for tibiotarsi widths are the same. This could be a genuine biological difference in the allometric relationship, but we feel that it actually reflects problems of measuring appropriate tibia widths and CSMI values in the older birds. As birds age the shape of the tibiatarsus changes, becoming less circular. This potentially increases CSMI for a given amount of bone. As we are assuming that tibiotarsi are circular, our approximate estimate of CSMI fails to capture this change. For this reason, we prefer to use body weight as a covariate to adjust for bone size/shape in the analyses of bone mechanical properties that follow.

Bone Porosity

Histological analysis of sections from all birds indicated the presence of primary osteonal canals throughout the cortex. In contrast to the control and restricted birds, both modern strains at 15 days of age were characterized by large oval-shaped canals extending from the periosteal surface (Fig. 2a, b). Morphometric analysis was unable to detect any differences in bone porosity in the endosteal area between bird groups or ages, or as a result of restricted feeding (Fig. 3a). Porosity in the periosteal area was reduced between 15 and 42 days ($F_{1,54} = 93.0$; $P < 0.001$), and was consistently lower in the control strain than the two modern strains ($F_{2,6} = 12.14$, $P < 0.01$; Fig. 3b). At 15 days, cortical bone from the restricted-fed selected birds had a much lower porosity than their *ad lib* counterparts. This was still true at 42 days, however, the

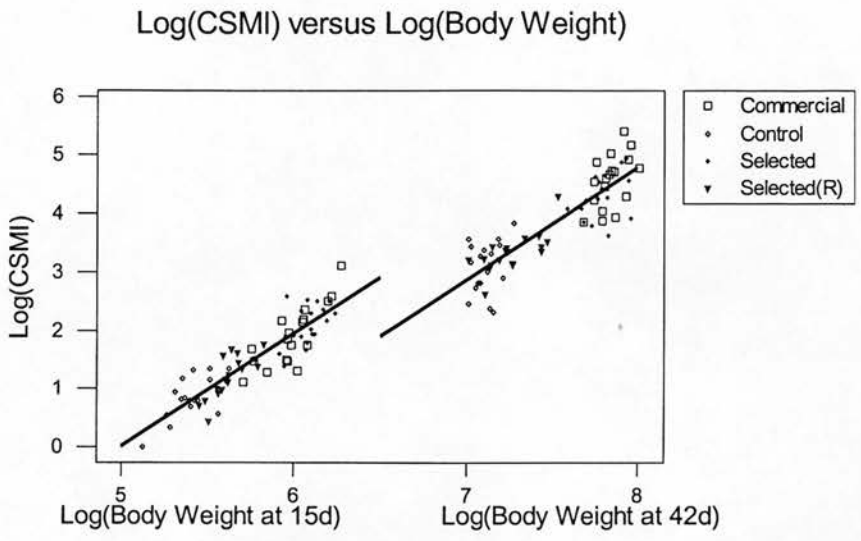


Fig. 1. Plot of log (CSMI) versus log (body weight) for 4 groups of birds at 15 and 42 days of age. The lines show different allometric relationships between the two variables for the 2 ages: y = −9.7 + 1.93x at 15 d, and y = −10.6 + 1.93x at 42 d.

difference was reduced (feeding.age interaction $F_{1,54}$ = 33.94, $P < 0.01$) due to the smaller reduction in porosity over the period 15–42 days seen in the restricted-fed birds when compared to the *ad lib*-fed birds.

Plasma Mineral Status and Bone Mineralization

There was no detectable effect of feeding regime or strain on total plasma Ca but it was increased with age (mean over all groups: 1.32 mg/dl at 15 days, 1.45 mg/dl at 42 days; SEM = 0.034, $F_{1,118}$ = 6.99, $P < 0.01$) whereas plasma inorganic P (mean over all groups: 7.29 mg/dl at 15 days, 5.19 mg/dl at 42 days; SEM of log (P) = 0.026, $F_{1,118}$ = 85.34, $P < 0.001$) was reduced with age. There were also no significant effects on circulating ionized Ca (mean over all groups: 1.48 mMol/l). At 15 days, total bone mineral content (% ash) was similar in all groups. It remained similar at 42 days for tibiotarsi from the control strain, but decreased in the two modern strains (strain.age interaction $F_{2,126}$ = 3.09, $P < 0.05$) (Fig. 4). In addition, restricted feeding considerably increased ash content in the bones of the selected strain by 42 days (feeding.age interaction $F_{1,126}$ = 17.01, $P < 0.001$). Bone Ca content did not vary among the groups, however, it did increase slightly with age (15 days = 15.1%, 42 days = 16.1%, SEM = 0.23, $F_{1,118}$ = 9.67, $P < 0.01$). Bone P content was also similar in all groups at 15 days (mean 8.06%), and increased slightly with age in all groups, although an age difference was only detected in the restricted-fed chicks (selected 8.27%, commercial 8.23%, control 8.73%, restricted-selected 9.0%; (SEM = 0.16; feeding.age interaction $F_{1,118}$ = 5.27, $P < 0.05$).

Bone Mechanical Properties

There was an increase in bone stiffness with age ($F_{1,126}$ = 2236.1, $P < 0.001$) (Fig. 5a), and the modern

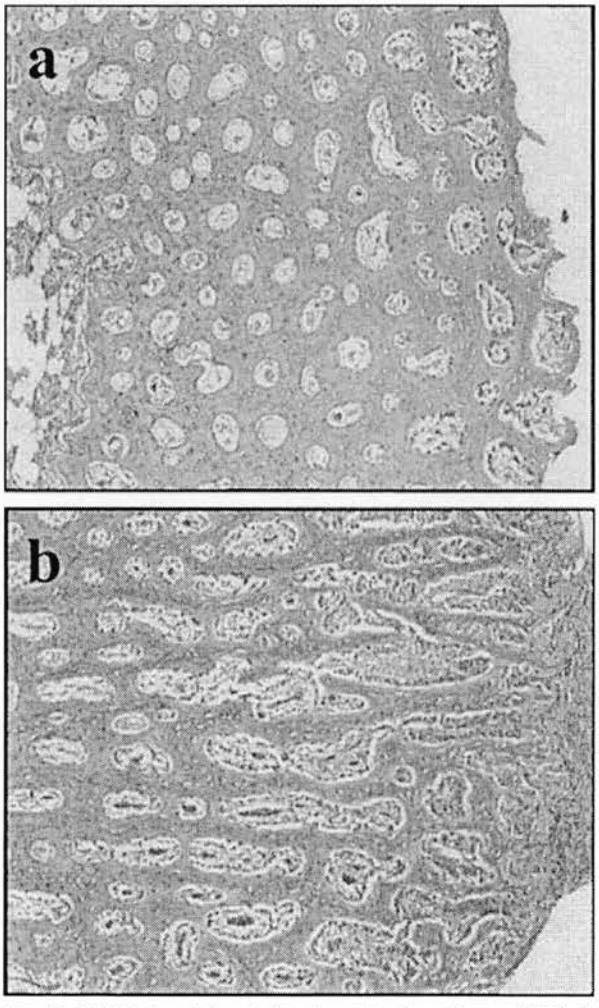


Fig. 2. Toluidine blue-stained cortical sections of 15-day-old birds showing differences in porosity and osteon shape within the periosteum of **(a)** control and **(b)** *ad lib* modern birds. In both micrographs the periosteal and endosteal surfaces are to the right and left, respectively. (Original magnification ×208).

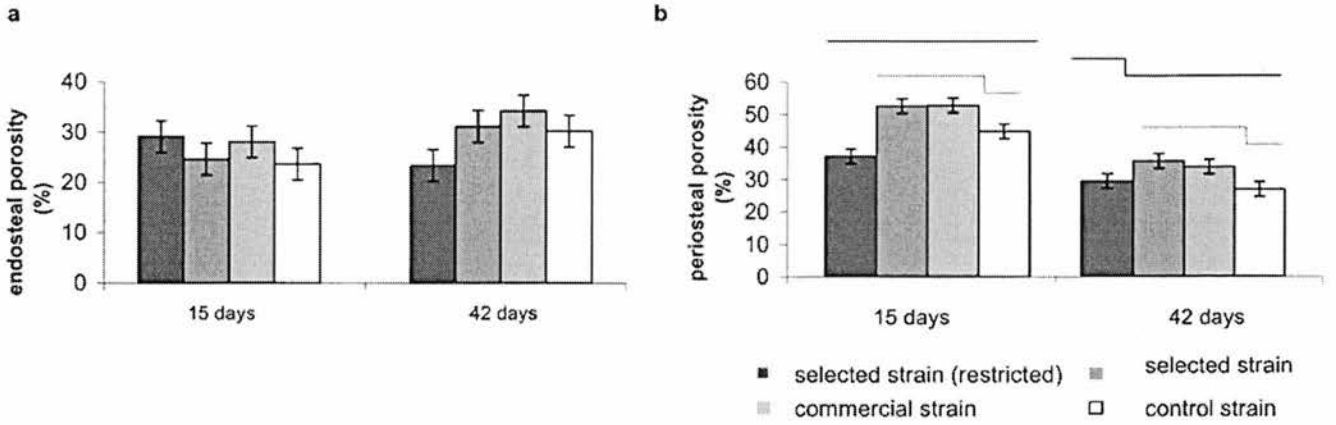


Fig. 3. Comparisons of **(a)** endosteal porosity and **(b)** periosteal cortical porosity between strains and feeding regimes at 15 and 42 days of age along with standard errors for comparison across ages. In **(b)**, lines symbolize average porosity levels for treatment comparisons: solid line, 15-day birds more porous than 42-day birds, the difference smaller for the restricted-fed selected strain; dashed line, modern strains more porous than control strain.

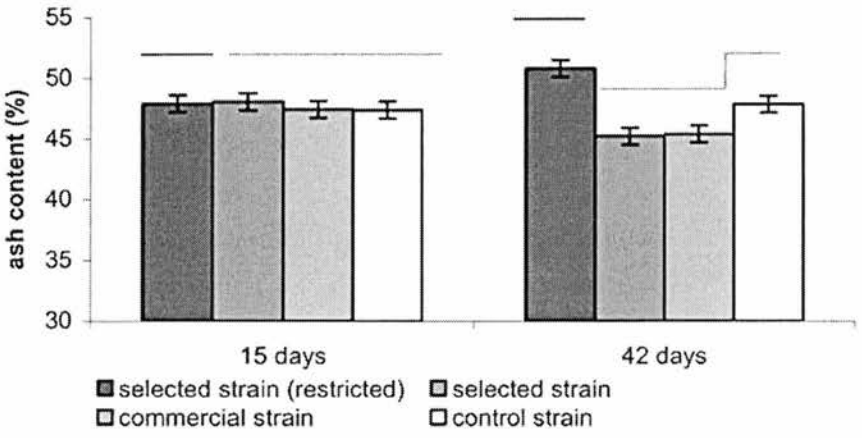


Fig. 4. Comparison of tibiotarsus ash content between strains and feeding regimes along with standard errors for comparison across ages at 15 and 42 days of age. The lines symbolize average % ash levels for treatment comparisons: solid line, the restricted fed-selected strain has increased % ash at 42 days, dashed line, amongst the *ad lib*-fed groups, the controls had similar % ashat both ages, while the modern birds had decreased % ash at 42 days.

strains had similar values, which were greater than those of the control strain, proportionately more so at 15 days than at 42 days ($F_{2,126} = 4.80$, $P < 0.01$). Restricted feeding reduced stiffness at 15 d, but not distinguishably at 42 days ($F_{1,126} = 6.09$, $P < 0.05$). The stiffness of the restricted-fed group and the controls were similar at both ages. Covariate analysis suggested that tibiotarsus stiffness corrected for log body weight was proportionately greater in the 2 slow-growing groups than those of the 2 faster-growing groups at 15 days but not at 42 days (15 d: fast = 46,000 N/mm, slow = 75,000 N/mm; $P < 0.01$; 42 d: fast = 297,000 N/mm, slow = 370,000 N/mm; NS; average SED on log scale = 0.086, df = 4) (Fig 5b). The effect of covariate adjustment is illustrated in Figure 6, where a single line for each age group has been drawn through the age-group means for log body weight and log stiffness. The slow-growing groups, lying to the left of these lines, had their stiffness values adjusted upwards, while the fast-growing groups, lying to the right, were adjusted downwards. The slopes were 1.74 (SE = 0.16) at 15 days and 0.65 (SE = 0.18) at 42 d. The differences in these two slopes is the reason for a separate correction for body weight at each age.

Maximum load increased with age ($F_{1,126} = 2402.1$, $P < 0.001$), and bones from the control strain were consistently weaker than those from the modern strains ($F_{2,6} = 42.87$, $P < 0.001$) (Fig. 5c). Bone strength was reduced in the selected strain by restricted feeding. This effect was proportionately larger at 15 days (feeding.age interaction $F_{1,126} = 7.48$) ($P < 0.01$). Covariate adjustment gave approximately equal maximum loads for fast- and slow-growing groups at 42 d, and proportionately greater maximum loads for slow-growing birds at 15 days (15 d: fast = 65 N, slow = 92 N, $P < 0.01$; 42 d: fast = 358 N, slow = 395 N; NS, averaged SED on log scale = 0.063, df = 4) (Fig. 5d). The slopes were 1.60 ($SE = 0.16$) at 15 d, and 0.72 ($SE = 0.16$) at 42 d.

Localization of Osteoblasts, Osteoclasts and Cement Lines

No obvious differences were apparent in the distribution of ALP and TRAP activity among groups of birds of the same age. At both 15 and 42 days, strong ALP activity

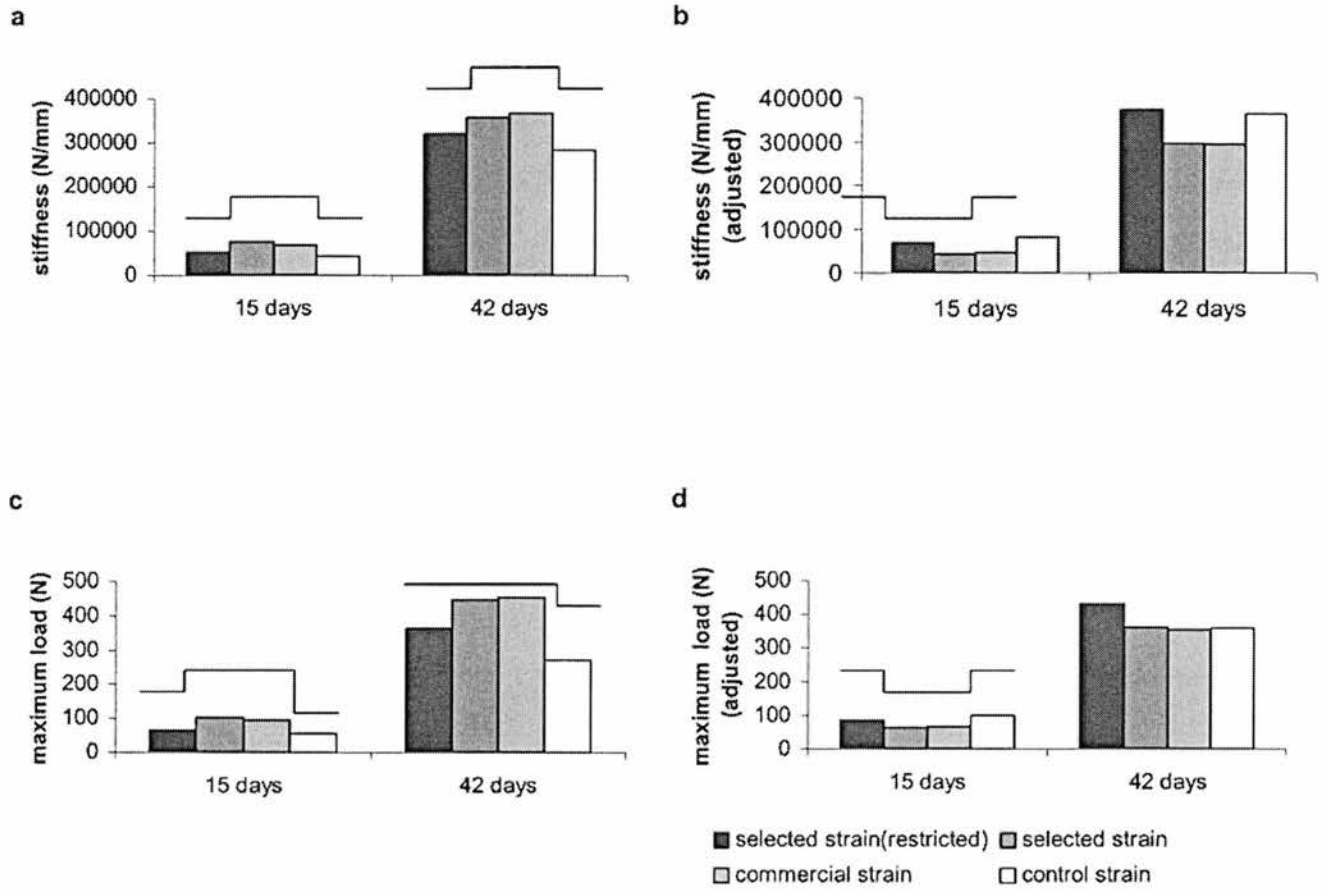


Fig. 5. Comparison of (**a**, **b**) tibiotarsus stiffness and (**c**, **d**) maximum load (strength) before (**a**, **c**) and after adjustment for body weight (**b**, **d**), between strains and feeding regimes at 15 and 42 days of age (standard errors on log scale given in text). The lines symbolize the average stiffness or maximum load treatment comparisons. (**a**) Stiffness increased between 15 and 42 days, always greater in the modern strains than the control strain. Restricted feeding reduced stiffness below *ad lib* levels at 15 days, but gave stiffness levels indistinguishable from either the *ad lib*-fed selected strain or the control strain at 42 days, (**b**) After correction for body weight differences at each age, the slow-growing groups had stiffer bones than the fast growing groups at 15 days. (**c**) Maximum load increased with age and was always proportionately greater in the fast-growing strains than the control strain, more so at 15 days. Restricted feeding reduced maximum load at 15 days to a level intermediate between the *ad lib*-fed selected group and the control group, but at 42 days maximum load became indistinguishable from the *ad lib*-fed group. (**d**) After correction for body weight differences at each age, the slow-growing groups had stiffer bones than the fast-growing groups at 15 days.

was found in osteoblasts associated with the periosteal surface and primary osteons in the periosteal and middle areas of the diaphyseal cortical bone. Less enzyme activity was noted to be associated with the endosteal surface and the canals within the endosteum (Fig. 7a). Endosteal surfaces reacted strongly for TRAP activity (Fig. 7b) as did surfaces within the occasional giant canal of the endosteal area of 42-day-old birds (Fig. 7b). There was no evidence of TRAP activity in osteonal canals in the periosteal area (Fig. 7c). In contrast, the remodelling pattern changed with group of bird and age. At 15 days of age, some of the canals situated within endosteal bone adjacent to the endosteal surface of control and restricted birds had evidence of cement lines (Fig. 7d,e) whereas no cement lines were detected throughout the cortical bone of both modern bird strains of this age. In contrast, by 42 days of age, birds from all groups revealed evidence of cement lines within endosteal and middle areas of bone but these were not present within periosteal bone (Fig. 7f).

Discussion

In a former study [1] we reported that in fast- and slow-growing birds periosteal porosity reduced with age. This is confirmed in the present work, where in addition, growth restriction was found to significantly reduce porosity, further highlighting the connection between the rate of body weight increase and cortical bone porosity. The increases in bone Ca and P content observed with age, and the differences observed in ash content among bird groups in the present study were also consistent with the results of our previous study [1]. By 42

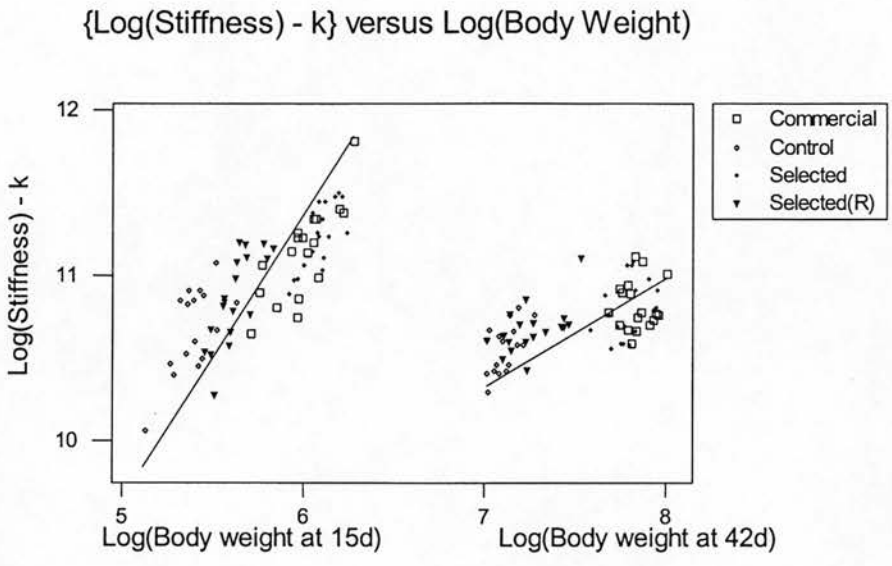


Fig. 6. Plot of {log(stiffness) − k} versus log(body weight) for birds of 15 days (k = 0) and 42 days (k = 2). Lines have the slopes for the covariate adjustments and pass through the overall age means: y = 0.9 + 1.74x at 15 d, and y = 5.7 + 0.65x at 42 d.

days, bones from the two slower-growing groups of birds had a much higher total ash content than those from the faster-growing groups.

A significant role for dietary mineral content as a limiting factor in bone ash content was not found in chicks during a previous experiment [11]. It is therefore unlikely that deficiencies in systemic mineral concentrations are a likely explanation for the observed lower bone ash content of the modern strains and this was also suggested in the present study where the fast-growing birds showed normal homeostatic levels of ionic and total Ca and inorganic P (based on the control strain). It is likely that the more rapid bone formation during modeling of cortical bone in the fast-growing modern birds is responsible for the observed lower bone ash content.

Bone width is of great importance in bone strength [17] and as expected the *ad lib*-fed modern birds had wider tibiotarsi than the control strain at both ages. Therefore, it is likely that in order to increase load capacity as quickly as possible, the fast-growing modern strains expanded tibiotarsi width more rapidly by increasing both periosteal apposition and the production of new osteons at the periosteal surface. It is therefore likely that the primary osteons of the modern birds have less time to infill by bone apposition before they are resorbed by expansion of the marrow cavity. The fast-growing modern birds appear unable to compensate for this by increasing primary osteon bone apposition rates, resulting in more porous bone. An explanation for the reduced osteonal bone apposition rates is not obvious but it is possible that increased transit times through the osteoblast lineage would account for the reduced primary osteon infilling. Alternatively, the reduced osteon infilling may be a transient phenomenon related to the high rate of bone formation at the periosteal surface.

Skeletal adaptation to load during growth and development involves the same fundamental mechanisms present in the adult skeleton but is more complex because of the interaction between the ongoing adaptation and the underlying growth process. Using computer models of the developing human skeleton it has been reported that up to the age of 5 years the periosteal apposition rate was high (> 10 μm/day) which included approximately equal contributions from the effects of both mechanical loading and biologically driven growth [7]. According to our hypothesis, the greater body weight of the faster-growing birds resulted in a load-induced increase in primary bone formation at the periosteal surface. This periosteal response is similar to that observed in other growing animals; in young beagle dogs the bones of immobilized forelimbs developed at a reduced rate and had a smaller cross-section than the bones of the normally loaded limb [18]. Under extreme conditions such as space flight-induced microgravity, reduced loading resulted in the total arrest of periosteal growth in growing rats, which resumed upon returning to earth [19]. Intriguingly, different cellular responses to *in vitro* loading occur in bones from fast- and slow-growing birds, suggesting that genetic selection for high growth rates results in a relative insensitivity to loading [20]. In apparent agreement with this premise are the studies of Patterson et al. [21] in which artificial weight loading of fast-growing chickens failed to have an effect on stress or elastic modulus of the tibiotarsi. However, as these authors did not monitor physical activity of their loaded and control birds, the actual loading on the birds over the period of the experiment was not known. The experiment described here also has this inherent weakness. There will be additional feeding activity in the ad libitum-fed selected birds compared to restricted-fed birds. However, restricted feeding itself tends to produce an increase in activity (P. Hocking, personal communication), and it is possible that the lighter control birds may be more active than the heavier modern strains.

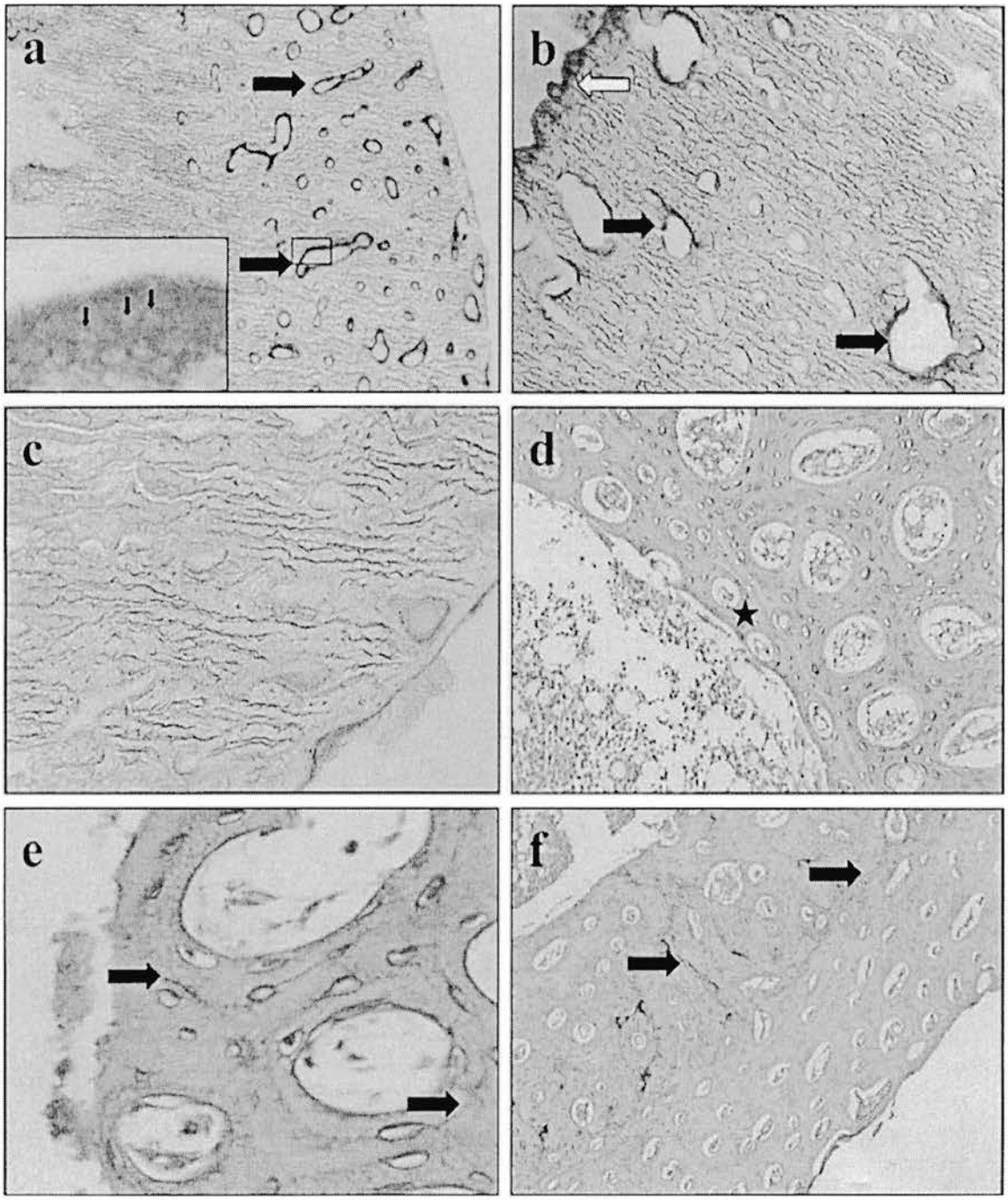


Fig. 7. Demonstration of sites of bone formation, resorption and remodelling between strains, feeding regimes at 15 and 42 days of age. (**a**) Alkaline phosphatase activity(arrows) within canals of the periosteum of a 42-day-old *ad lib* modern bird. Inset is a higher magnification of the boxed area showing osteoblasts (arrows) situated within in the alkaline phosphatase-positive endosteal region, (**b**) acid phosphatase activity at the endosteal surface (white arrow) and within canals of the endosteum (black arrows) of a 42-day-old *ad lib* modern bird, (**c**) lack of acid phosphatase activity at the periosteal surface of a 42-day-old *ad lib* modern bird, (**d**) low power micrograph of a toluidine blue-stained section of a 15 day-old slow-growing control bird. The area adjacent to the endosteal surface containing remodelling osteons is marked (*). (**e**) High-power micrograph of cement lines (arrows) of remodelling osteons shown in an equivalent area, marked (*) in **d**, (**f**). Toluidine blue-stained section showing cement lines (arrows) of remodelling osteons in the endosteal bone of a 42-day-old control bird. In all micrographs the periosteal and endosteal surfaces are to the right and left, respectively. (Original magnification (a & f) ×208, (a-inset) ×2860, (b, c, d) ×419, (e) ×1676.

Therefore, we are not certain of the relative amount of activity among the bird groups. With this caveat, our data support and extend previous conclusions [20] that some skeletal adaptations to load fail in fast-growing birds and appear to fail 'less' when the fast-growing birds are growth restricted.

Previously, it was uncertain whether the increase in periosteal porosity observed in modern broiler strains was due to increased bone resorption or reduced bone formation [1]. The complete lack of osteoclastic activity and cement lines in the periosteum of all birds clearly indicates that remodelling of primary osteons was not taking place. Therefore, the difference in periosteal porosity between the fast- and slow-growing birds is likely to be due to the rapid formation of primary osteons at the periosteal surface and incomplete infilling, by osteoblasts, of the resultant canal. A role for bone resorption in the development of endosteal porosity, however, cannot completely be ruled out as strong evidence for the remodelling of osteons in this area was observed in all 42-day-old birds and further quantitative studies are required. It was of further interest to note that cement lines were also noted in the 15-day restricted and control birds but not in the modern fast-growing birds and this may reflect differences in the speed of bone marrow expansion between the groups of birds.

The poor bone quality observed in the faster-growing selected strain is unlikely to be due to a genetic predisposition, since slowing its growth rate produced tibiotarsi of similar dimensions, porosity and mechanical properties to those of the control strain. Furthermore, when adjusted to average body weight of contemporaries, bones from the slower-growing control strain and the restricted-fed modern birds were at least as strong and stiff as those from the fast-growing *ad lib*-fed birds. This was probably due to higher cortical porosity and lower mineral content achieved by the *ad lib*-fed birds, at all ages. A variable related to fracture potential—work to failure—was not measured in this study. However, such data would be of interest in this context as there are instances in which strength and stiffness of bone increases but the energy required to fracture is reduced. Our results are at variance with those of LeTerrier et al. [4] who found that growth restriction did not improve tibiotarsi quality in chickens although biomechanical properties or porosity were not measured. However, these authors used only a 28% reduction of dietary energy and protein contents, and it is possible that the growth restriction was not large enough to produce a detectable effect.

The slopes of the log (body weight) covariates for stiffness (1.74 at 14 d and 0.65 at 42 d) and maximum load (1.60 at 14 d and 0.72 at 42 d) differ markedly for the 2 ages, and bracket the values found for adult birds from a wide range of wild species, 1.1 for stiffness and 0.98 for maximum load [22]. Comparisons with domestic species, selected to eat and grow more or fed to grow less quickly, may not be particularly valid. However, if reliable, our experimental data suggest that one model of bone-body similarity will not adequately describe bone strength at 2 ages, as two different models are needed for wing and leg bones in wild birds [23]. Regrettably, we do not have the data combining bone dimensions and orientation which would allow calculation of the measures of stress and modulus of elasticity which Patterson et al. [21] found successful in correcting for bone size differences. A further consequence of our presumed difficulty in accurately measuring CSMI is that we cannot correct our stiffness measurements for bone dimensions accurately enough to examine the more subtle relationships between stiffness and porosity or mineralization [24, 25].

In studies with cortical bone from bovine tibias, the collagen fiber orientation ranked highly as a predictor of bending properties, with density and mineralization being the next best predictors. Similarly, meat-type birds fed a low calcium and phosphorus diet had weaker, less rigid bones [21] whereas no relationship with mineralization was noted in birds from a wide range of wild species although their level of mineralization was unclear [22]. Differences in the relative contribution of various histocompositional variables to the bending properties of plexiform and osteonal bone were noted, but in osteonal specimens 88.0% of elastic modulus variability was accounted for by bone ash content and collagen fiber, orientation [26, 27]. We cannot say whether collagen fiber orientation differed between the fast- and slow-growing birds, thereby contributing to the altered bending properties between the strains. Its relative importance in comparison to porosity in growing broiler bone is, however, unclear due to the very high density of primary osteons within the newly deposited bone.

In conclusion, fast growth resulted in rapid bone deposition at the periosteal surface, which was regulated by the growth rate, and not the genetic predisposition, of the bird. This rapid circumferential expansion was accompanied by increased porosity resulting from the rapid incorporation of primary osteons at the periosteal surface and the incapacity of osteoblasts to completely infill the resultant canal. The increased porosity, together with reduced mineralization, are likely explanations for the poorer mechanical properties of tibotarsi observed in the fast-growing birds. This may help to explain the increased fracture risk in fast-growing animals and further understanding of these events will form the basis of the prevention of fractures during phases of rapid growth.

Acknowledgments. The authors thank Elaine Seawright and Bob Fleming for technical assistance and Ross Breeders Ltd, John Thomson and Sons Ltd, BBSRC and DEFRA for funding.

References

1. Williams B, Solomon S, Waddington D, Thorp B, Farquharson C (2000) Skeletal development in the meat-type chicken. Brit Poultry Sci 41:141–149
2. Farquharson C, Jefferies D (2000) Chondrocytes and longitudinal bone growth: the development of tibial dyschondroplasia. Poultry Sci 79:994–1004

3. Kestin SC, Knowles TG, Tinch AE, Gregory NG (1992) Prevalence of leg weakness in broiler chickens and its relationship with genotype. Vet Rec 131:190–191
4. LeTerrier C, Rose N, Constantin P, Nys Y (1998) Reducing growth rate of broiler chickens with a low energy diet does not improve cortical bone quality. Brit Poultry Sci 39:24–30
5. Wolff J (1892) Das Gesetz der transformation der Knochen – the law of bone remodelling (1986). Maquet P, Furlong R (translators) Springer-Verlag, Berlin
6. Lanyon LE (1980) The influence of function on the development of bone curvature. An experimental study on the rat tibia. J Zool London 192:457–466
7. Carter DR, Wong M, Orr TE (1991) Musculoskeletal ontogeny, phylogeny, and functional adaption. J Biomechanics 24:3–16
8. van der Meulen MCH, Beaupre GS, Carter DR (1993) Mechanobiologic influences in long bone cross-sectional growth. Bone 14:635–642
9. Martin RB, Burr DB, Sharkey NA (1998) Skeletal tissue mechanics. Springer-Verlag, New York
10. Thorp BH, Waddington D (1997) Relationships between the bone pathologies, ash and mineral content of long bones in 35-day-old broiler chickens. Res Vet Sci 62:67–73
11. Williams B, Waddington D, Solomon S, Farquharson C (2000) Dietary effects on bone quality and turnover, and Ca and P metabolism in chickens. Res Vet Sci 69:81–87
12. Banks WJ (1986) Applied veterinary histology (2nd ed). Williams and Wilkin, Baltimore, Hong Kong, London, Sydney, pp 119–145
13. Bell KL, Loveridge N, Jordan GR, Power J, Constant CR, Reeve J (2000) A novel mechanism for induction of increased cortical porosity in cases of intracascapular hip fracture. Bone 27:297–304
14. Bell KL, Loveridge N, Reeve J, Thomas CDL, Feik SA, Clement JG (2001) Super-osteons (remodelling clusters) in the cortex of the femoral shaft: influence of age and gender. Anat Rec 264:378–386
15. Fleming RH, McCormack HA, McTeir L, Whitehead CC (1998) Medullary bone and humeral breaking strength in laying hens. Res Vet Sci 64:63–67
16. Farquharson C, Whitehead CC, Rennie S, Thorp B, Loveridge N (1992) Cell proliferation and enzyme activities associated with the development of avian tibial dyschondroplasia: an in situ biochemical study. Bone 13:59–67
17. Schwartz SM, Biewener AA (1992) Shape and scaling. In: Biewener AA (Ed.) Biomechanics, structures and systems: a practical approach. Oxford University Press pp 21–43
18. Uhtoff HK, Jaworski ZFG (1978) Bone loss in response to long-term immobilisation. J Bone Jt Surg 60B:420–429
19. Morey ER, Baylink DJ (1978) Inhibition of bone formation during space flight. Science 201:1138–1141
20. Pitsillides AA, Rawlinson SCF, Moseley JR, Lanyon LE (1999) Bone's early response to mechanical loading differs in distinct genetic strains of chick: selection for enhanced growth reduces skeletal adaptability. J Bone Miner Res 14:980–987
21. Patterson PH, Cook ME, Crenshaw TD, Sunde ML (1986) Mechanical properties of the tibiotarsus of broilers and poults loaded with artificial weight and fed various dietary protein levels. Poultry Sci 65:1357–1364
22. Cubo J, Casinos A (2000) Mechanical properties and chemical composition of avian long bones. Eur J Morphol 38:112–121
23. Cubo J, Casinos A (1998) Biomechanical significance of cross-sectional geometry of avian long bones. Eur J Morphol 36:19–28
24. Schaffler MB, Burr DB (1988) Stiffness of compact bone: effects of porosity and density. J Biomechanics 21: 13–16
25. Currey JD (1988) The effect of porosity and mineral content on the Young's modulus of elasticity of compact bone. J Biomechanics 21:131–139
26. Martin RB, Ishida J (1989) The relative effects of collagen fiber orientation, porosity, density, and mineralization on bone strength. J Biomechanics 22:419–426
27. Martin RB, Boardman DL (1993) The effects of collagen fiber orientation, porosity, density, and mineralization on bovine cortical bone-bending properties. J Biomechanics 26:1047–1054